

Application and Characterization of Anaerobic Ammonium
Oxidation (Anammox) Process to Treat Sidestream and
Mainstream Wastewaters: Lab-scale and Full-scale Studies

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Abstract

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Compared to conventional nitrification and denitrification, anaerobic ammonium oxidation (anammox) is a more energy saving and cost effective process for biological nitrogen removal (BNR). To date, the anammox process has been applied widely and designed mainly to treat sidestream wastewaters. However, only 15%-20% of the influent domestic sewage nitrogen loading is present in the sidestream, while the bulk of it still needs to be removed from the mainstream. Research efforts thus have shifted from sidestream to mainstream applications of anammox, including the application of anammox bioreactors at low temperature, low influent ammonium strength, and under the presence of organic carbon (characteristic of municipal mainstream wastewaters). In this dissertation research, the applicability of anammox process in lab-scale and full-scale mainstream systems have been studied. The overall goals of this dissertation research were (1) to develop an effective strategy to enrich an anammox moving bed biofilm reactor (MBBR) under low influent nitrogenous substrate concentration and ambient temperature (23 C°), and link microbial ecology to the process performance of the enriched anammox MBBR; (2) to explore the catabolism and anabolism of anammox bacteria in a mainstream MBBR before and after dosing of organic carbon;

(3) to extend the strategy of mainstream anammox enrichment under ambient temperature (23 C°) to low temperature (15 C°) , and link microbial ecology to the process performance; (4) to evaluate the microbial community structure, kinetics and performance during startup and long-term operation of a full-scale mainstream anammox process; (5) to investigate the reliability of the new enriched mainstream anammox MBBR under the imposition of additional wet weather flow; (6) to develop a reliable and sensitive method of hydrazine determination in anammox reactor.

First, an anammox MBBR was successfully enriched under low nitrogenous substrate and ambient temperature. It needs to be addressed that, even with the limited fraction of *Candidatus* “Kuenenia stuttgartiensis” in the coming inoculum from the sidestream MBBR, *Candidatus* “Kuenenia stuttgartiensis” was effectively enriched in the biofilm biomass of the mainstream MBBR. Moreover, the enhanced activity of *Candidatus* “Kuenenia stuttgartiensis” was demonstrated through this whole time series experiments, and achieved the most competitive level among all functional groups. Therefore, the importance and necessity of bioaugmentation are addressed during the enrichment of mainstream anammox process.

Second, successful enrichment of a mainstream anammox moving bed biofilm reactor was accomplished at low nitrogenous substrate and low temperature. 16S amplicon sequencing was employed to investigate the microbial ecology of the biomass in the biofilm and suspension. Results showed the dominance of *Candidatus* “Kuenenia” related anammox bacteria in the biofilm of mainstream reactor, though *Nitrospira* spp. related nitrite oxidizing bacteria were still detected in a limited fraction. These results

are crucial to show the effective enrichment of anammox reactor by bioaugmentation even under low temperature, especially in a practical way.

Third, the performance, kinetics and microbial ecology were studied before, during and after the imposition of additional organic carbon. The dosing of organic carbon resulted in a reversible negative impact on both the activity of AMX and the reactor performance. Stable isotope probe and 16S amplicon sequencing were applied to investigate the metabolism of functional groups. The results showed anammox bacteria are not capable of assimilating acetate, while the community assimilating ^{13}C -labeled acetate was mainly assigned to denitrifiers. Presence of denitrifiers were observed in the mainstream MBBR and stayed inactive without sufficient organic carbon. In sum, these results demonstrate that the mainstream anammox process as tested was resilient to a short period imposition of organic carbon.

Fourth, the performance and microbial ecology of the ambient-temperature mainstream anammox were investigated under wet weather condition. Based on the full recovery of reactor performance as well as the stable microbial ecology, the applicability of the mainstream MBBR under wet weather conditions was demonstrated.

Fifth, real-time polymerase chain reaction was applied to evaluate the startup and operation of two parallel sidestream DEMONTM systems as well as the initiation of the mainstream anammox process through bioaugmentation. Results provided the evidence that anammox bacteria was the most abundant functional group in two parallel DEMONTM systems, showing the successful startup in the sidestream.

Furthermore, anammox bacteria were selectively retained in the mainstream with high bioaugmentation rates from the sidestream. These results are critical to demonstrate the significance of bio augmentation in the startup of mainstream anammox system even in full-scale wastewater water treatment plant.

Finally, a sensitive and reliable spectrophotometric method was proposed to measure hydrazine concentration in anammox reactor. The concentration of hydrazine could be precisely determined in the presence of nitrite, when certain amount of sulfamic acid is introduced.

In sum, the application and characterization of anaerobic ammonium oxidation (anammox) process to treat sidestream and mainstream wastewaters in both lab-scale and full-scale was investigated in detail. From a practical perspective, the knowledge gained can lead to a better design and operation of engineered nitrogen removal process.

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List of Abbreviations

16S rRNA	16S ribosomal RNA
AMO	Ammonia Monooxygenase
AMX	Anaerobic Ammonia Oxidizing Bacteria
AOB	Ammonia Oxidizing Bacteria
BNR	Biological Nitrogen Removal
CMX	Complete Ammonia Oxidizing Bacteria
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
FA	Free Ammonia
FISH	Fluorescent in situ hybridization
HAO	Hydroxylamine Oxidoreductase
HRT	Hydraulic Retention Time
LPM	Liter per Minute
MBBR	Moving Bed Biofilm Reactor
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
NGS	Next-Generation Sequencing
NOB	Nitrite Oxidizing Bacteria
OHO	heterotrophic organisms
<i>p</i> -DAB	<i>p</i> -dimethylaminobenzaldehyde
RBCOD	Readily biodegradable COD
SBCOD	Slowly biodegradable COD
SRT	Solids Retention Time
WWTP	Wastewater Treat Plant
AMO	Ammonia Monooxygenase

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Chapter 1. Introduction

1.1 General introduction

Wastewater is used water. In homes, wastewater includes water from sinks, showers, bathtubs, toilets and washing machines. Businesses and industries also produce their share of wastewater that should be properly treated before discharging into water bodies. Also, wastewater includes storm runoff, which contains harmful substances that wash off roads and rooftops [1]. Because of concerns about wildlife habitats and human health, it is essential to treat wastewater properly.

Wastewater treatment is a process used to clean wastewater into effluent stream that can be either recycled to the water cycle with limited environmental issues or reused. The physical infrastructure used for wastewater treatment is called a “wastewater treatment plant” (WWTP). Combined physical, chemical, and biological processes will be applied to remove soluble and particulate compounds that may represent a risk to public health or environment [2].

1.2 Conventional wastewater treatment

Processes employed in different WWTPs vary based on local regulation and diverse composition of sewage. But in a typical wastewater treatment plant treating municipal sewage, following steps will be mostly applied: preliminary treatment, primary treatment, secondary treatment, sidestream treatment, tertiary treatment, disinfection, and solids handling [2].

The objective of preliminary treatment is to remove big solids and other large materials, mostly inert. Removal of these materials is a necessary step to ensure the

efficient operation and maintenance of subsequent treatment units. In preliminary treatment, operations typically include coarse screening and grit removal. Usually, bar screens, which come in a variety of shapes and sizes, are used for coarse screening. In grit chambers, the velocity of the water through the chamber is maintained sufficiently high to prevent the settling of most organic matters. Flow measurement devices, often standing-wave flumes, are usually included at the preliminary treatment stage.

The main objective of primary treatment is to remove organic and inorganic solids from sedimentation, and those materials that will float by skimming as well. Approximately 25 to 50% of the incoming chemical oxygen demand (COD), 50 to 70% of the total suspended solids (SS), and around 65% of the oil and grease are removed from this unit [3]. Moreover, some organic nitrogen, organic phosphorus, and heavy metals associated with solids are also settled and removed during primary treatment, but colloidal materials and dissolved substances are barely affected. In large wastewater treatment plants, primary sludge is most commonly processed biologically by anaerobic digestion [4]. In the digestion process, anaerobic and facultative bacteria metabolize the organic material under high temperature and long hydraulic retention time (HRT), thereby reducing the final disposal volume.

Effluent from primary treatment flows into secondary treatment for further treatment, mainly removal of residual COD and SS. In most WWTPs, secondary treatment follows primary treatment and involves the removal of biodegradable organic matter and nitrogenous compounds using aerobic-anaerobic biological treatment processes.

Microorganisms are produced during this biological treatment process, which need to be separated from the treated wastewater by sedimentation to produce clarified secondary effluent. The unit for sedimentation used in secondary treatment, often termed as secondary clarifier, operates similarly as the primary clarifier. In most WWTPs, part of the biological solids separated from secondary clarifier is recycled to keep sufficient microbial organism in the process, the left is normally combined with primary sludge for sludge digestion.

Disinfection typically involves the application of a chlorine solution or other chemicals or even ultraviolet radiation

The primary and secondary sludge streams produced are most commonly treated through anaerobic digestion. Anaerobic digestion is a collection of processes by which microorganisms break down biodegradable material, mainly sewage sludge in this case, in the absence of oxygen. Gas containing about 60% methane is produced during this process and can be recovered as an energy source [5]. Instead of being converted to biogas, bulk of the assimilated nitrogen is converted to ammonium contained in the effluent from an anaerobic digester. This nitrogen-rich effluent, containing around 1000 mg-N/L ammonium, could be further treated by biological processes. This microbial nitrogen removal process after the anaerobic digester is where anammox was initially found and mostly applied nowadays [6, 7].

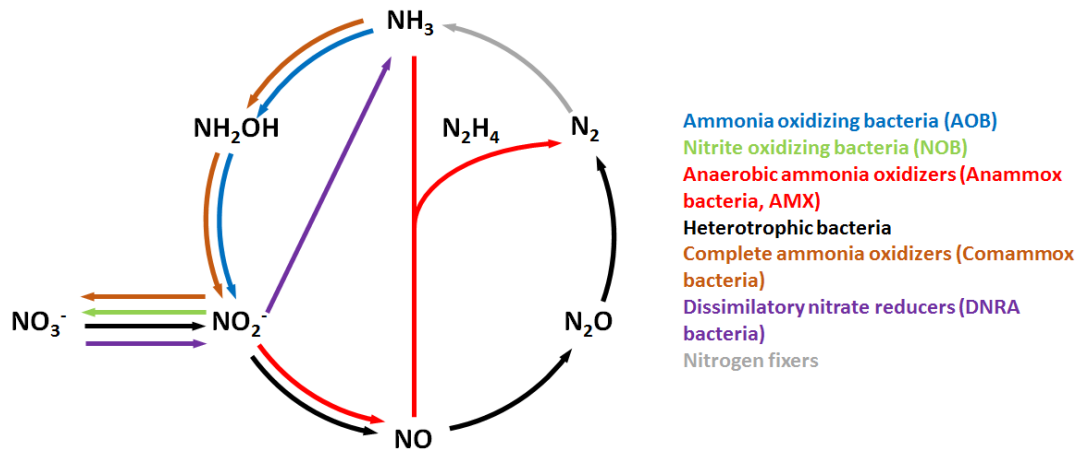


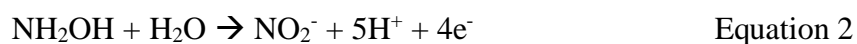
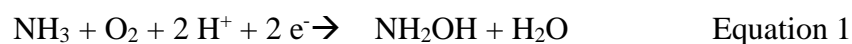
Figure 1 Nitrogen cycle

1.3 Conventional biological nitrogen removal in WWTP

Nitrogen removal from wastewater treatment is of great value, because of the significant adverse environmental impact of ammonia/ ammonium on a receiving water body, such as eutrophication. In WWTPs, conventional biological nitrogen removal (BNR) is accomplished in two steps: nitrification, carried out by AOB and NOB, denitrification carried out by denitrifiers. Nitrification is a biological oxidation of ammonia to nitrite and subsequently to nitrate by AOB and NOB, respectively. These two distinct types of nitrifying bacteria are both chemolithoautotrophs, and use carbon dioxide as their only carbon source for growth. Denitrification is a biological reduction of ionic nitrogen oxides such as nitrate and nitrite to dinitrogen gas using organic carbon as the electron donor [8].

1.3.1 Ammonia oxidation

The ammonia oxidation by AOB includes two reactions. First, ammonia is oxidized to hydroxylamine using oxygen as the electron donor, in which the key enzyme is ammonia monooxygenase (AMO) (Equation 1). AMO is a transmembrane copper protein to catalyze the conversion of ammonia to hydroxylamine. Then hydroxylamine is further converted to nitrite by hydroxylamine oxidoreductase (HAO) (Equation 2) [9, 10]. Four electrons are discharged in the second reaction, two of which are provided for the electron input of the first reaction, whereas the rest is delivered to the terminal oxidase through an electron transport chain, to produce a proton motive force.



Four genera contain AOB (*Nitrosomonas*, *Nitrospira*, and *Nitrosovibrio*, and *Nitrosococcus*) [11], among which *Nitrosomonas* (AOB) is most commonly studied and observed in municipal wastewater system. *Nitrosomonas* has an optimum pH of 6.0-9.0 and a temperature of 20-30°C [12-14].

1.3.2 Nitrite oxidation

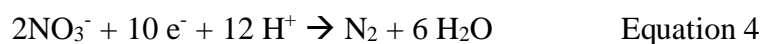
Nitrite produced in previous step is oxidized to nitrate by nitrite oxidoreductase (NXR) of NOB [15, 16] (Equation 3).



Four genera contain NOB (*Nitrobacter*, *Nitrococcus*, *Nitrospira*, *Nitrotoga* and *Nitrospira* [17-21]. Among these identified NOB, *Nitrotoga*, *Nitrospira* and *Nitrobacter* are most commonly studied and observed in municipal wastewater system. [20-24]. A recent study showed *Nitrospira* spp. have not just higher affinities for nitrite and oxygen, but also a higher biomass yield and energy transfer efficiency relative to *Nitrobacter* spp. [25]. In a word, compared with conventionally studied *Nitrobacter* and *Nitrotoga* related NOB, *Nitrospira* related NOB are more abundant at low temperature in mainstream processes of wastewater treatment plants.

1.3.3 Denitrification

Produced nitrate from nitrification can be reduced in anoxic condition to ultimately produce dinitrogen gas (N₂) (Equation 4).



This respiratory process of denitrification reduces oxidized forms of nitrogen in response to the oxidation of an electron donor, mainly organic matter from wastewater in this case. The process is performed primarily by common heterotrophic bacteria (HOB), although autotrophic denitrifiers including anaerobic ammonia oxidizing bacteria (abbreviated, anammox, AMX) have also been identified [26-28].

1.4 Anaerobic oxidation of ammonium

Nitrogen removal from wastewater treatment is of great value because of the significant adverse environmental impact of ammonia/ammonium on receiving water body, such as eutrophication. For decades, nitrogen removal in WWTPs is mostly

performed by nitrification and denitrification, both of which are costly and energy consuming. In the biological removal of nitrogen and organic matter, provided aeration accounts for about 50% of electricity consumption in WWTPs. After nitrification process, denitrification is accomplished by organic carbon as the electron donor, and extra organic carbon is commonly required to ensure a low nitrate residual in the effluent. Furthermore, this conventional process has an additional environmental impact because of high biomass production [29] and greenhouse gas emission, especially N_2O [30, 31]. As a recently established technology, anammox process is an environmental friendly, cost effective biological nitrogen removal process. Removal of nitrogen by anammox is based on completely autotrophic oxidation of ammonium with nitrite as the electron acceptor, which means reduced sludge production and consumption for aeration and no organic carbon is needed [32-34] since only half of the ammonium is oxidized. Overall, anammox process can reduce sludge production by 80–90% as compared to conventional nitrification-denitrification process [35, 36].

Nevertheless, the application of anammox process for nitrogen removal was not straightforward. The first obstacle needs to overcome was the slow growth rate of the responsible microbial community in both lab-scale and full-scale systems [37]. In lab-scale systems, this trouble was conquered by using systems with improved biomass retention such as sequencing batch reactors (SBR) [38]. At industrial scale, the first full-scale anammox reactor was started for the treatment of rejection water at the sludge treatment plant in 2002 [39]. In that case, up-flow anammox reactor was

applied and coupled with a partial nitrification reactor termed SHARON, which produces a mixture of ammonium and nitrite for subsequent anammox reaction. Later on, the first one-stage anammox called CANON (Completely Autotrophic Nitrogen removal Over Nitrite) system, was initiated at the wastewater treatment plant in Strass, Austria [40]. Instead of two individual reactors in SHARON-anammox system, partial nitrification and anammox processes happen in the same reactor under limited aeration condition [41]. Autotrophic nitrogen removal is currently applied widely for the treatment of a variety of ammonium-rich municipal and industrial wastewaters: tannery, food-processing, semiconductor, fermentation, yeast, distillery, winery industries [7, 42]. Among all these applications, most of them were implemented with high strength ammonia wastewater treatment in sidestream in municipal WWTPs [7].

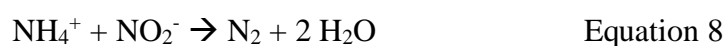
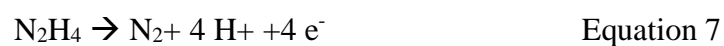
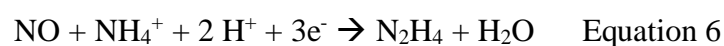
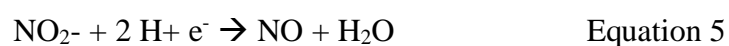
1.4.1 Advantages of anammox process

Aeration supplied in nitrification and organic substrates provided in denitrification determine that the conventional nitrogen removal process is remarkably energy consuming and associated with the excess production of sludge. Instead, anammox bacteria are capable of converting ammonium and nitrite directly to N_2 without additional oxygen; therefore, this process does not require aeration and excess electron donors. Overall, in partial nitrification/anammox systems, oxygen demand is significantly reduced because oxidation of ammonium only needs to be performed partially instead of complete conversion to nitrate when applying conventional nitrogen removal process. A low biomass yield is provided by the autotrophic process

of anammox bacteria and AOB, thus a considerable reduction of sludge production can be achieved. Overall, application of the anammox process in wastewater treatment could lead to a cost effective and environmental friendly operation. The Strass wastewater treatment plant used nitrification/anammox to treat reject water, resulting in a decrease in energy consumption from 2.66 to 1.50 kWh per kg N removed [43].

1.4.2 Molecular mechanism of anammox

To date, including newly found *Candidatus* “*Brocadia caroliniensis*” [44], eleven anammox bacteria species belonging to five genera have been identified: *Candidatus* “*Brocadia*”, “*Kuenenia*”, “*Scalindua*”, “*Anammoxoglobus*” and “*Jettenia*”. All catabolic processes of anammox metabolism occur in the anammoxosome [45], which is cell compartment bound by a single curved membrane and constitutes 50-70% of the total cell volume [37, 44, 46].



Anaerobic ammonium oxidation has three steps with nitric oxide and hydrazine as intermediates. The first step is the reduction of nitrite to nitric oxide by nitrate reductase (NirS). Produced nitric oxide and ammonium are combined by hydrazine

synthase (HZS) to form hydrazine [47]. Soluble cytochrome-c proteins in the anammoxosome lumen provide the electrons required for these two steps, 30% of the protein complement are cytochrome c type proteins [48]. The last step is oxidation of hydrazine to dinitrogen gas through hydrazine oxidoreductase (HDH) [49]. The electrons formed via hydrazine oxidation are transferred to another 4-electron cytochrome-c protein, which brings these electrons to ubiquinone in the anammoxosome membrane. Then these electrons are delivered to cytochrome-bc1 in the membrane, which then transfers them to the soluble cytochrome-c proteins that originally supplied NirS and HZS with electrons, restoring them as electron donors. In short, cytochrome-bc1 is a proton pump, translocating 6 protons into the anammoxosome lumen for every 4 electrons transferred [48].

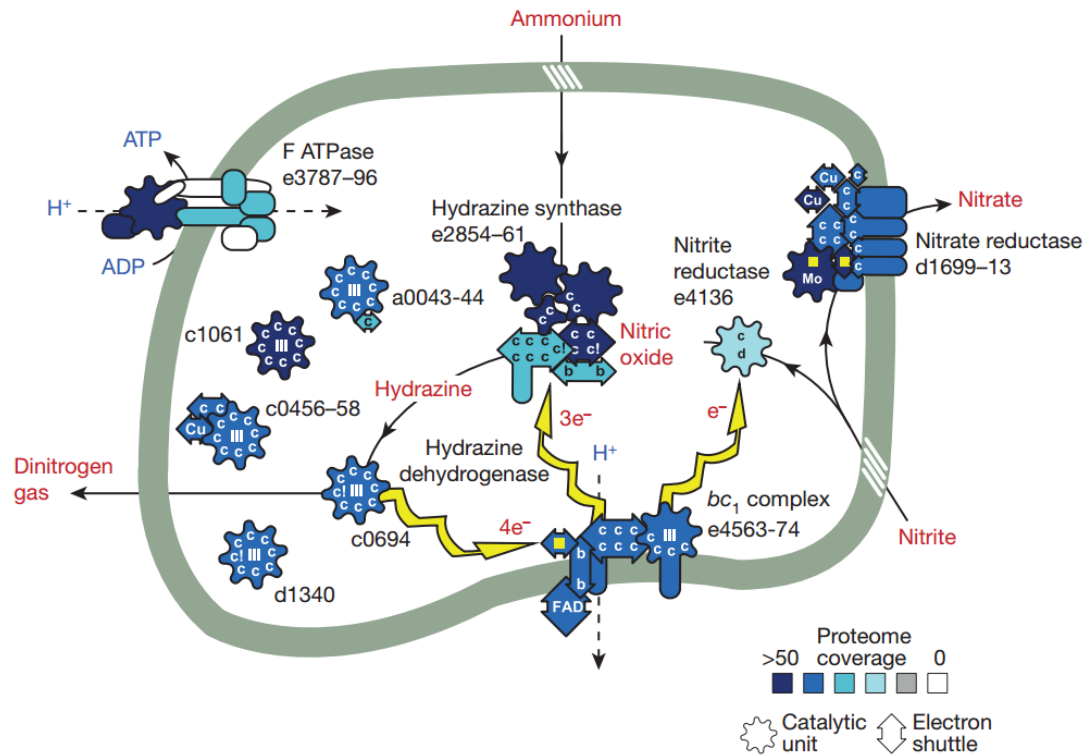


Figure 2 Biochemical pathway and enzymatic machinery of *Candidatus "Kuenenia stuttgartiensis"*[48]

1.4.3 Temperature and rainfall influence on anammox

Anammox bacteria were detected in a broad range of temperatures. The existence of anammox bacteria in extreme temperature environments was proved by evidence, such as in hot springs (up to 80 °C) and marine sediments down to -5 °C [50-52].

It was reported that enriched anammox bacteria were recovered from marine sediment that had an optimal temperature of 25 °C [53], and even an optimal temperature down to 12 °C [54]. Furthermore, loss of activity of anammox marine species was observed at 37 °C [55]. However, for those anammox bacteria found and applied in wastewater treatment systems, the optimal temperatures are between 30 °C and 40 °C [56-58].

Batch tests were applied to perform the activity test of anammox between 10 °C and 45 °C, and both biofilm and granular biomass were tested. A similar pattern of increasing anammox activity was monitored up to 40 °C, and inhibition of activity emerged at 45 °C [59]. Analogous inhibitions of anammox activity were reported by other researchers above 40 °C [60, 61]. Biomass from anammox systems operated at different temperatures (10, 20 and 30 °C) was applied to perform anammox activity at 10, 20 and 30 °C[62]. It was reported that at low temperature the adapted anammox biomass had a relatively low reduction in activity [62]. Furthermore, it was reported that an anammox sequencing batch reactor was successfully operated under 18 °C with an inorganic nitrogen removal rate equal to 0.325 g N L⁻¹d⁻¹ (a decrease from 0.6 g N L⁻¹d⁻¹ at 30 °C) after a step decrease in temperature from 30 °C, this research also provided the evidence that anammox biomass gradually adapted to low temperature has higher specific anammox activity at low temperature compared with a non-adapted one [59].

It has been reported that a reliable anammox process could be maintained at room temperatures in single-stage reactors with relatively high nitrogen removal [63, 64]. Successful operation of the anammox process was also researched down to 15 °C [65-67], but a considerable reduction of anammox activity was observed below 15 °C [66]. Moreover, researchers have achieved a total nitrogen removal of 39% during the treatment of synthetic wastewater at a temperature down to 10 °C [68]. An anammox single-step moving bed biofilm reactor (MBBR) was applied to treat synthetic

wastewater from 20 °C to 10 °C. However, after the drop of temperature in this operation, the nitrogen removal rate was observed to be reduced over 60% [69].

1.4.4 Organic matter influence on anammox

Anammox bacteria are chemoautotrophic microorganisms capable of oxidizing ammonium and reducing nitrite to dinitrogen gas, and use inorganic carbon (CO₂) as the only carbon source [70]. The concentration of bicarbonate in the influent of the Anammox process is therefore particularly important for the cultivation of Anammox bacteria [70, 71]. It was observed that anammox bacteria were far more vulnerable than other functional groups in a biofilm nitrification-anammox process under limited inorganic carbon conditions, both biomass concentrations and nitrogen consumption rates were reduced [71]. Moreover, build-up and retention of NOB were monitored in the biofilm-based nitrification-anammox process in longer-term even after inorganic carbon sufficient conditions [71].

It is generally considered that organic matter has adverse effects on either biomass concentration or activity of anammox bacteria [72-74].

Inhibition of anammox activity was observed with the presence of high concentrations of organic matter [74]. However, low concentrations of organic matter had no significant negative impact on the anammox process [75, 76]. It seems the impact of organic matter on anammox process is concentration based.

Under high concentrations of the organic matter condition, heterotrophs could be quickly enhanced, especially in anoxic conditions. Overgrowing of OHO could

compete with AMX and reduce the overall performance and reliability of the anammox process [73, 75, 77, 78]. However, in some conditions, the anammox bacteria are still the dominant species in the systems even with the presence of high concentration of organic matter, but the anammox bacteria utilize organic matter rather than ammonium and nitrite as a substrate [75, 79, 80]. Thus, a decrease of nitrogen removal and reduction of anammox activity could be observed.

In different applied wastewater treatment processes, the COD/N ratio in the influent of anammox process could vary between 1/1 and 10/1 [40, 81, 82]. It was commonly reported that anammox bacteria co-exist with heterotrophic bacteria. In certain conditions, the co-existence of anammox bacteria with heterotrophs is beneficial in achieving good nitrogen removal performance under conditions of high concentrations of organic matter [76, 83, 84].

It was shown that propionate and acetate can probably be utilized by anammox bacteria, and after they were fed with propionate, there was no significant change in fractions of anammox bacteria and heterotrophs [75]. It was also reported that the anammox bacteria *Candidatus* “Anammoxoglobus propionicus” could out-compete other anammox bacteria when propionate was supplied as the organic carbon [79]. However when inorganic carbon was still the only carbon source for anammox bacteria, assimilation of propionate was not observed. [80]. Moreover, anammox bacteria can be enriched in the presence of acetate, and the enriched biomass can oxidize other kinds of organic matter [80].

In an anammox sequencing batch reactor, the anammox community was significantly influenced by the presence of acetate, *Candidatus* “*Brocadia fulgida*” was accumulated to become the most dominant anammox species, which is probably due to its ability to oxidize acetate [85]. There was no negative influence on *Candidatus* “*Brocadia fulgida*” activity when it was monitored after a switch of organic matter from acetate to glucose. This suggests that *Candidatus* “*Brocadia fulgida*” can successfully compete with heterotrophs in the presence of other types of organic matter [85].

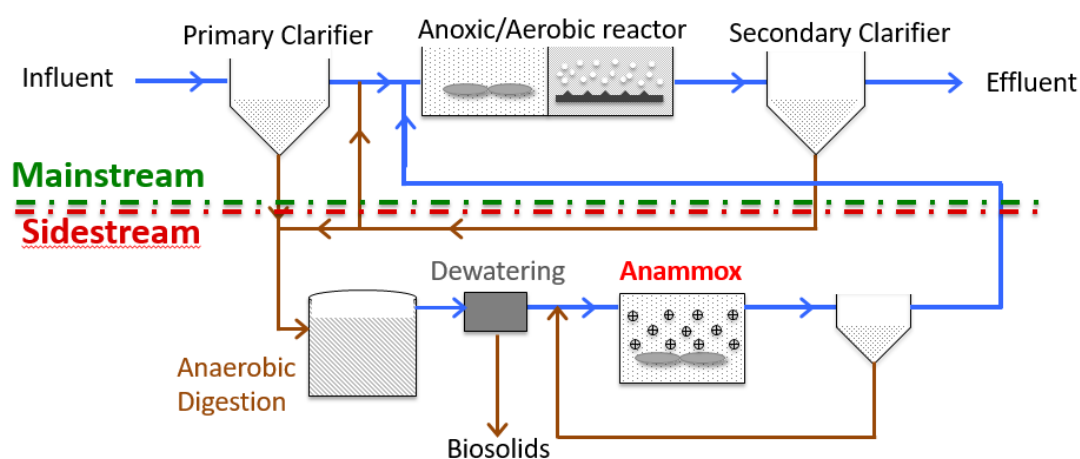


Figure 3 Configuration of conventional wastewater treatment plant

Therefore, application of anammox process need to focus on the out-selection of *Nitrospira* spp. rather than other NOB. Furthermore, nitrification was generally considered to be carried by two separate groups of microorganisms. However, complete oxidation of ammonia into nitrate by a single microorganism was predicted in 2006 [86]. In 2015, the presence of such organisms was discovered within the *Nitrospira* genus [87, 88].

1.5 Research hypotheses and objectives

1.5.1 Enrichment of the mainstream anammox MBBRs at ambient and low temperature

Successful operation of anammox has previously been reported in ambient temperature [63, 64] as well as in low temperature [66, 68, 69, 89]. However, enrichment of an anammox reactor at low nitrogenous substrates and low temperature only through bioaugmentation has not been well explored. It was hypothesized here that, by coupling with a well operated sidestream anammox MBBR, the mainstream anammox reactor could be effectively enriched at ambient temperature and low temperature. Moreover, the microbial community of mainstream MBBR was expected to be analogous to that of the coupled sidestream MBBR, especially in the suspension due to constant bioaugmentation. The specific objectives were to: 1) track the performance and kinetics of the mainstream MBBR during enrichment to evaluate the activity of AMX; 2) explore the composition of microbial ecology of the suspended and biofilm biomass in the mainstream MBBR as well as the sidestream MBBR.

1.5.2 Impact of organic carbon and wet weather flow on mainstream deammonification system

Previously, a mainstream anammox MBBR was successfully enriched at low substrates and ambient temperature conditions through continuous bioaugmentation from a sidestream system, though the viability of mainstream deammonification under other mainstream conditions was yet to be determined. It was hypothesized here that imposition of organic carbon and wet weather on the mainstream MBBR could result

in a shift on the kinetics of functional groups as well as the overall microbial community. The principal goal of this study was to determine the impact of additional organic carbon on the microbial ecology and performance of a mainstream anammox MBBR. The specific objectives were to: 1) investigate the deviation of the total bacteria structure in both biofilm and suspended biomass in response to the imposition of and recovery from organic carbon dosing and wet weather flow; 2) explore the catabolism and anabolism of the functional groups in the mainstream MBBR before and after the exposure to the additional organic carbon.

1.5.3 Long-term monitoring of full-scale mainstream and sidestream deammonification processes

The principal objective of this study was to characterize the performance and microbial ecology of a full-scale process that transitioned from conventional towards mainstream-deammonification based BNR, during the course of over two years. Specific objectives were to track the extent of nitrogen removal and the relative microbial concentrations as a function of operating conditions in the treatment process as well as the hydrocyclones, which were instituted to selectively enrich for AOB and anammox, while concurrently out selecting NOB.

1.5.4 Hydrazine measurement in anammox system

Hydrazine is a critical intermediate of anammox process. It has been reported that the determination of hydrazine by spectrophotometric method [90] could be interfered by nitrite, the electron acceptor for anammox reaction. In this paper, based on the

spectrophotometric method developed by George W. Watt [172], an upgraded method is proposed for the determination of hydrazine in the presence of nitrite.

The dissertation consists of eight chapters.

Chapter 1 presents the introduction to conventional wastewater treatment, conventional biological nitrogen removal, and anammox process.

Chapter 2 presents the reactor performance and molecular microbial ecology of a coupled mainstream (ambient temperature)-sidestream anammox system.

Chapter 3 describes the reactor performance and molecular microbial ecology of a coupled mainstream (low temperature)-sidestream anammox system.

Chapter 4 describes the influence on reactor performance and shift of molecular microbial ecology on an ambient temperature anammox MBBR before, during and after introducing organic carbon. And explore the catabolism and anabolism of AMX by applying stable isotope probe (SIP).

Chapter 5 demonstrates the reliability of a mainstream anammox MBBR under wet weather conditions.

Chapter 6 assesses the startup of two full-scale sidestream reactors and evaluate the application of the full-scale mainstream enrichment by applying molecular analysis.

Chapter 7 proposes a method to precisely measure the concentration of hydrazine in the presence of nitrite.

Chapter 8 presents the key findings and future directions.

Chapter 2. Enrichment and Characterization of an Anammox Moving Bed Biofilm Reactor (MBBR) to Treat Mainstream Wastewaters at Ambient Temperature

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2.1 Introduction

Nitrogen removal from wastewater treatment is of great value because of the significant adverse environmental impact of ammonia/ ammonium on the receiving water body, such as eutrophication. For decades, nitrogen removal in wastewater treatment plants (WWTPs) is mostly performed by nitrification and denitrification, both of which are costly and energy consuming. In the biological removal of nitrogen and organic matter, the provided aeration accounts for most of the electricity consumption in WWTPs [91]. After nitrification process, denitrification is accomplished by organic carbon as the electron donor and extra organic carbon is commonly required to ensure a low nitrate residual in the effluent. Furthermore, this conventional process has an additional environmental impact because of high biomass production [29] and greenhouse gas emission, especially N_2O [30, 31]. As a recently established technology, anaerobic ammonia oxidation is an environmental friendly, cost-effective biological removal process [56, 70]. Removal of nitrogen by anammox is based on completely autotrophic oxidation of ammonium with nitrite as the electron acceptor, which means reduced consumption for aeration and no organic carbon is needed, because only half of the ammonium is oxidized [32-34]. Anammox process has received a great deal of attention and recognized as an attractive alternative nitrogen removal process. Since it was discovered in 1995, anammox has been extensively implemented with high strength ammonia wastewater treatment in sidestream. However, as sidestream only accounts for 25% of total nitrogen loading in

WWTPs, more attention has been attracted to the implementation of anammox process in the mainstream.

Though more than 100 anammox applications are successfully operated worldwide [7], the study of anammox implementation in the mainstream is still limited due to the great difference in wastewater characteristics and operational conditions. Unlike the high strength ammonium wastewater in sidestream with warm circumstance (30-38°C), mainstream wastewater has much lower nitrogen concentrations (30-60 mg NH_4^+ -N/L) with relatively low temperature (below 30°C usually) [3]. Low temperature and low nitrogen concentration reduce the activity of AMX, which impacts process performance. With less free ammonia, the activity of NOB is promoted, which compete with AMX for nitrite resulting in more nitrate production. A very large decrease in specific anammox activity has been reported after lowering the temperature of reactors operated at warm temperatures [92-94]. To date, most studies focus on the impacts of mainstream operational conditions to already enriched anammox reactors. There is rarely a research study on the enrichment of a completely new mainstream anammox reactor through bioaugmentation.

In this study, a startup and enrichment methodology of lab-scale anammox MBBR was presented for treating low nitrogen load wastewater at ambient temperature. The specific objectives were to: 1) investigate the performance and kinetics of both MBBRs to evaluate the activity of AMX in the enrichment phase; 2) investigate the composition of microbial ecology of the suspension and biofilm in the coupled mainstream-sidestream system.

2.2 Materials and methods

2.2.1 Lab-scale reactors configuration and operation

A 6 L lab-scale MBBR was operated as the mainstream reactor. Mainstream MBBR was initiated with inoculation of 600 Kaldnes K1 biocarriers from a 6 L lab-scale sidestream anammox MBBR and additional 1400 virgin Kaldnes K1 biocarriers achieving a total volumetric fill capacity of 33%. Mainstream enrichment operation was divided into two phases. During phase A, mainstream MBBR was operated in sequencing batch mode with partial aeration from day 1 to day 81 at N-loading of $45 \pm 0.57 \text{ g NH}_4^+\text{-N/m}^3\text{/d}$. During operation of the mainstream MBBR in batch mode (Phase A), operation of the mainstream MBBR was stopped everyday for 2 hours until the thorough settlement of suspended biomass and biocarriers, then the influent containing substrate (NH_4^+) and trace elements was introduced manually. Different aeration strategies were applied to provide the optimal amount of oxygen for nitrification as described below (Table 1).

Table 1 Aeration pattern in batch mode mainstream MBBR

	Aeration rate (LPM)	Aeration time (on/off)
Phase A I (day 1-9)	0.1	5 s/95 s
Phase A II (day 10-18)	0.4	5 s/95 s
Phase A III (day 19-81)	0.1	1 s/99 s

During phase B, to speed up the enrichment of AMX, mainstream MBBR was connected with sidestream MBBR from day 82 (Figure 4). Prior to the transfer of 600

carriers into mainstream MBBR as inoculum, sidestream MBBR was operated at 35°C with 2000 Kaldnes K1 biocarriers (33% v/v fill) at a hydraulic retention time (HRT) of 1.5 days, achieving a nitrogen removal of $88 \pm 2.2\%$ at an influent N-loading of $0.65 \pm 0.03 \text{ kg NH}_4^+-\text{N}/\text{m}^3/\text{d}$. After the inoculation, 600 virgin kaldnes K1 biocarriers was added into sidestream MBBR immediately. Given that 600 mature carriers were removed from the sidestream MBBR, N-loading was thus decreased to $0.51 \pm 0.03 \text{ kg NH}_4^+-\text{N}/\text{m}^3/\text{d}$ from day 34 for a quick and sufficient recovery as well as the optimization of effluent ammonium and nitrite for the mainstream anammox MBBR.

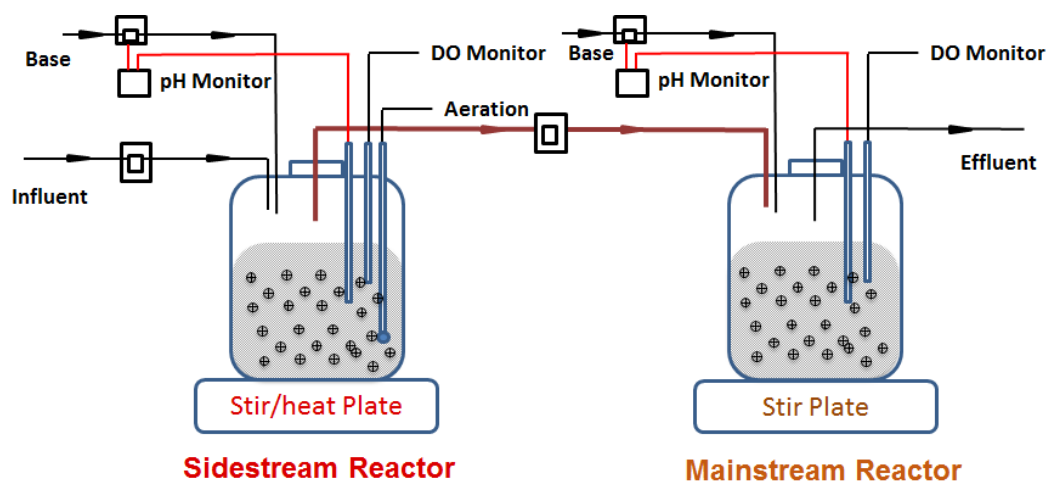


Figure 4 Set up of coupled sidestream/mainstream system

Reactor performance was monitored by measuring the concentrations of ammonia (potentiometry, Thermofisher, PA), nitrite (diazotization-colorimetry, RICCA, IN), nitrate (potentiometry, Fisher, MA) and hydroxylamine (colorimetry) [95] each sampling day. Reactor dissolved oxygen (DO) concentrations were measured by Clark type electrodes (05643-00, Cole Parmer, IL), interfaced to a DO monitor (01972-00, Cole Parmer, IL). Biomass concentrations in the biofilm and suspension

were measured in biofilm phase and suspension phase with Hach high-range COD digestion vials (20-1500 mg/L, Hach Inc., CA).

2.2.2 Sample collection and DNA extraction

Biomass samples from suspension and biofilm were collected at each sampling day and stored at -80°C for subsequent molecular processing. For suspension biomass sampling, 30 mL suspension from each reactor was taken and transferred into a 50 mL sterile tube (Corning, MA) using 50 mL sterile pipet. After centrifugation (Beckman Coulter, NJ) at 4 °C and relative centrifugal force of 8000G for 10 minutes, the supernatant was discarded and biomass was resuspended in 1 mL RNA protect reagent (Qiagen, CA). For biofilm biomass sampling, one carrier was sacrificed in each reactor and replaced with a virgin carrier. Biofilm from a single carrier was carefully and completely scraped with a sterile pipet tip and resuspended in 2 mL RNA protect reagent (Qiagen, CA), then it was visually checked to ensure no biofilm residual on the carrier. A Motor (Fisher, MA) with sterile tip (USA Scientific, FL) is applied for the disaggregation of detached biofilm biomass.

DNA was extracted using Qiacube with the DNeasy mini kit (Qiagen, CA) and extracted DNA was tested by Nanodrop Lite spectrophotometer (Thermo Scientific, WI) to ensure good DNA quality for the following molecular experiments.

2.2.3 Amplicon sequencing

Using the Illumina sequencing platform chemistry and protocols, 16S rRNA regions were targeted and amplified for analysis from both DNA and RNA extracted from

each reactor over the course of enrichment (Illumina Inc, San Diego, CA). The 1.66 GB of 16S rRNA genomic (DNA) sequences and 2.99 GB of 16S rRNA sequences from reactor cDNA were processed using the mothur software program (version 1.36.1) [96]. Contigs were formed using the paired Illumina reads and filtered based on size (275-325 bp), homopolymeric regions (maximum 8 bp), and ambiguous regions (maximum 0 bp). Mothur software was applied for post run bioinformatics analysis of the amplicon sequences.

2.2.4 Efficiency of functional groups

To explore the microbial community, extracted DNA was amplified and purified for 16S rRNA amplicon sequencing. The activity of different functional groups was estimated by performing 16S rDNA amplicon sequencing, using the amplified and purified cDNA from reverse transcription of RNA. To better evaluate the efficiency of different functional groups, a K ratio was calculated. Percentage of reads assigned to target functional group in genus level of 16S rDNA amplicon sequencing is applied as the numerator, while percentage of reads assigned to target functional group at the genus level in 16S rRNA amplicon sequencing is applied as the denominator.

$$K_{Nitrosomonas} = R_{Nitrosomonas(RNA)} / R_{Nitrosomonas(DNA)}$$

$$K_{Nitrospira} = R_{Nitrospira (RNA)} / R_{Nitrospira (DNA)}$$

$$K_{Candidatus \text{ "Kuenenia" }} = R_{Candidatus \text{ "Kuenenia" }} (RNA) / R_{Candidatus \text{ "Kuenenia" }} (DNA)$$

$R_{Nitrosomonas(RNA)}$, $R_{Nitrospira (RNA)}$, $R_{Candidatus "Kuenenia" (RNA)}$ = Percentage of reads assigned to functional groups at the genus level in 16S rDNA amplicon sequencing

$R_{Nitrosomonas(DNA)}$, $R_{Nitrospira (DNA)}$, $R_{Candidatus "Kuenenia" (DNA)}$ = Percentage of reads assigned to functional groups at the genus level in 16S rRNA amplicon sequencing

2.3 Results and discussion

2.3.1 Process performance of mainstream MBBR in batch mode

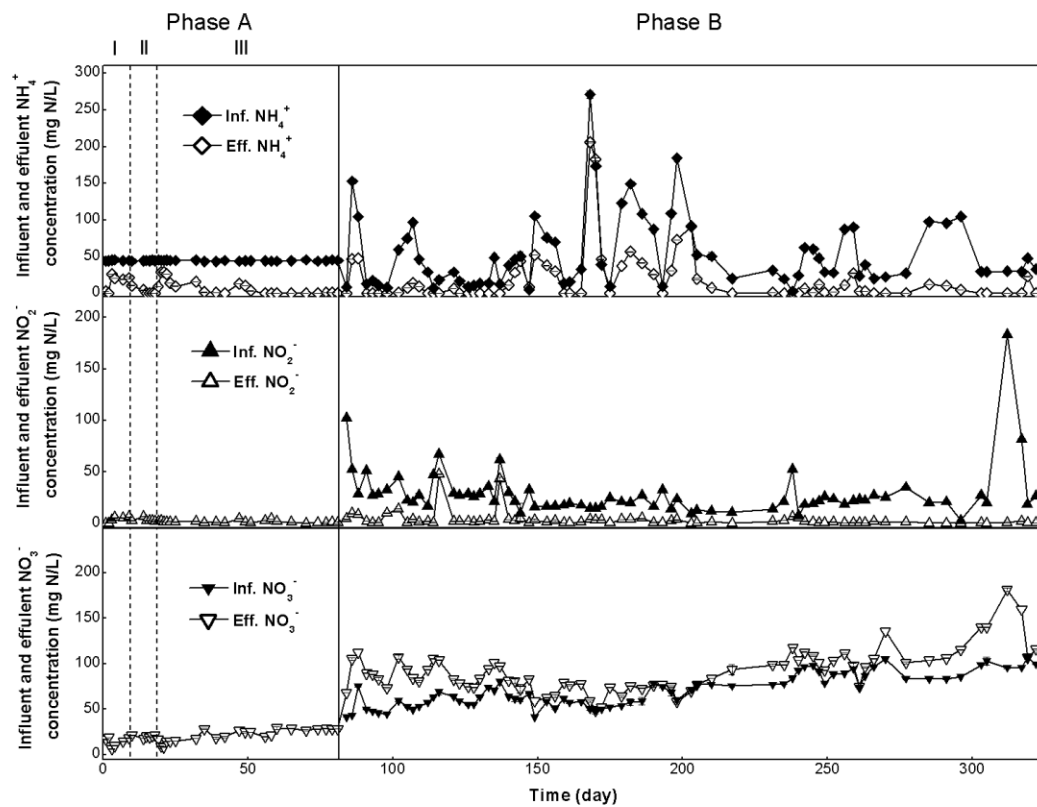


Figure 5 Performance of the mainstream MBBR; results represent average and standard deviation of duplicate measurements.

After initiation, only limited ammonium was oxidized under 5 s/95 s aeration mode in Phase A I, the effluent contained 22 ± 3.4 mg-N/L ammonium and 5.0 ± 1.3 mg-N/L,

resulting in an average total nitrogen removal of $12 \pm 11\%$ (Figure 5). To improve overall nitrogen removal performance, a higher aeration rate of 0.4 LPM under 5 s/95 s mode was employed in Phase A II. An average total nitrogen removal of $49 \pm 2.0\%$ was achieved; effluent contained 0.14 ± 0.06 mg-N/L of ammonium, 2.0 ± 0.38 mg-N/L of Nitrite and 21 ± 1.0 mg-N/L of nitrate. An increase of nitrate production was observed during the first 18 days; thus, a lower aeration rate was applied to improve overall anammox performance. In Phase A III, even with low aeration rate at 0.1 LPM under 1 s/99 s on/off mode, there was still relatively high nitrate production and no biomass was visually observed attached on the biocarriers.

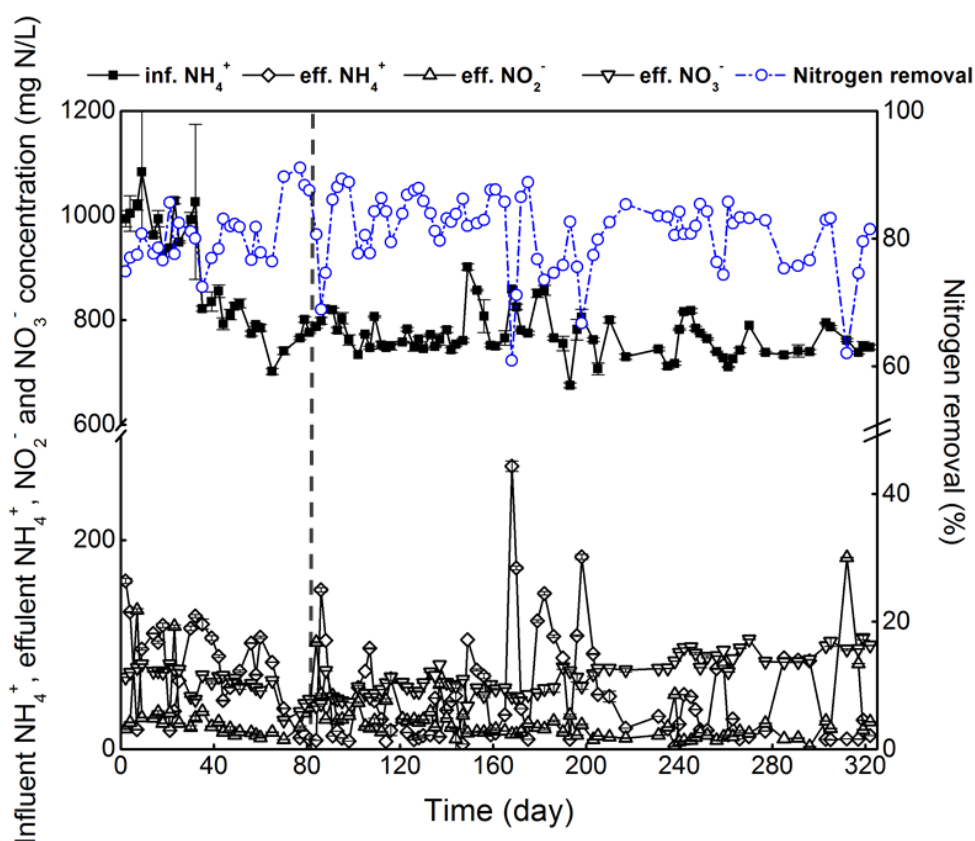


Figure 6 Performance of the sidestream; results represent average and standard deviation of duplicate measurements.

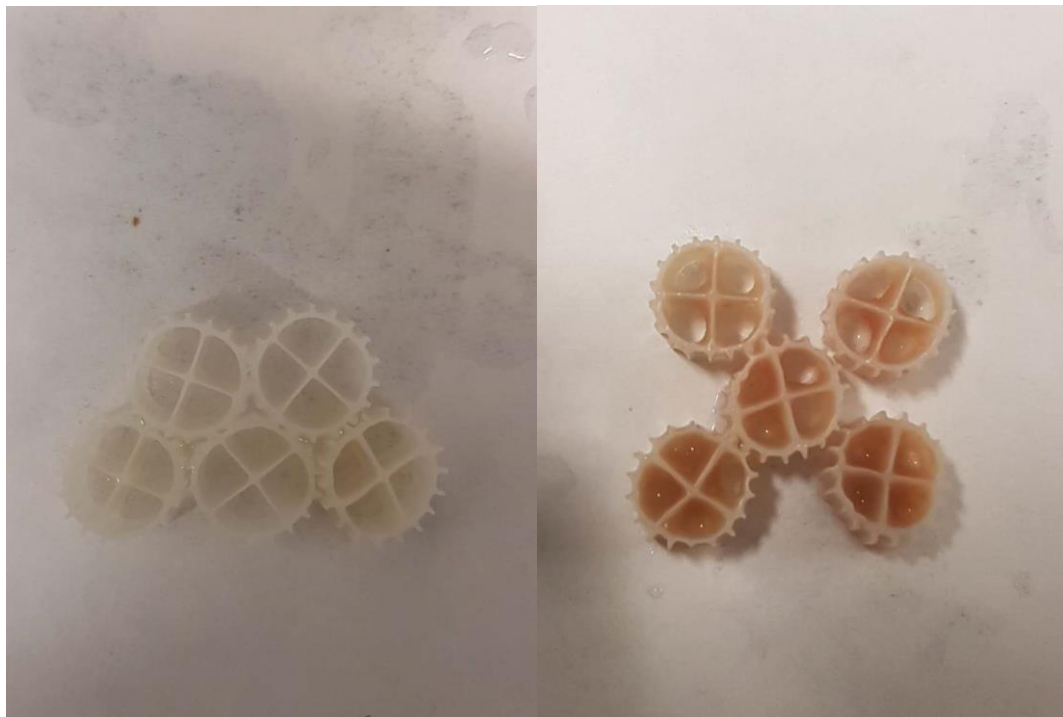


Figure 7 Biocarriers from the mainstream MBBR at day 0 (left) and day 322 (right)

During phase B, to speed up the enrichment of AMX, mainstream MBBR was connected with sidestream MBBR from day 82 (Figure 4). The sidestream MBBR was operated continuously at an influent N-loading of $0.65 \pm 0.03 \text{ kg NH}_4^+\text{-N/m}^3\text{/d}$, achieving an average total nitrogen removal of $88 \pm 2.2\%$ in 33 days. However, the loss of 600 mature biocarriers resulted in a shock on the operation of the sidestream MBBR, thus the N-loading was adjusted to $0.51 \pm 0.03 \text{ kg NH}_4^+\text{-N/m}^3\text{/d}$ to provide a more stable and low concentrations of substrates (ammonium and nitrite) for the mainstream MBBR,

From day 82, the sidestream MBBR was connected to the mainstream MBBR. After seeding with biocarriers in the mainstream MBBR, with nitrogen load of 0.51 kg

$\text{NH}_4^+\text{-N}/\text{m}^3/\text{d}$, the effluent of sidestream contained 49 ± 6.0 mg-N/L ammonium and 27 ± 3.0 mg-N/L nitrite, representing an average nitrogen removal of $81 \pm 3.1\%$ (Figure 6). Compared with the average nitrogen removal of $88 \pm 2.2\%$ at nitrogen load of 0.65 kg $\text{NH}_4^+\text{-N}/\text{m}^3/\text{d}$ before removing those 600 carriers, sidestream MBBR showed a slightly worse process performance. Actually, the mass of functional microorganism in the suspension was extremely lower than in biofilm carriers [71], therefore the removed 600 carriers reduced almost 30% of total functional microorganism in sidestream MBBR. Moreover, attachment of biomass on virgin carriers is an extraordinarily time consuming process, months was taken to have the same amount of biomass in those late adding carriers. It would result in a more vulnerable reactor during this period, which can also tell from fluctuant process performance in the sidestream MBBR.

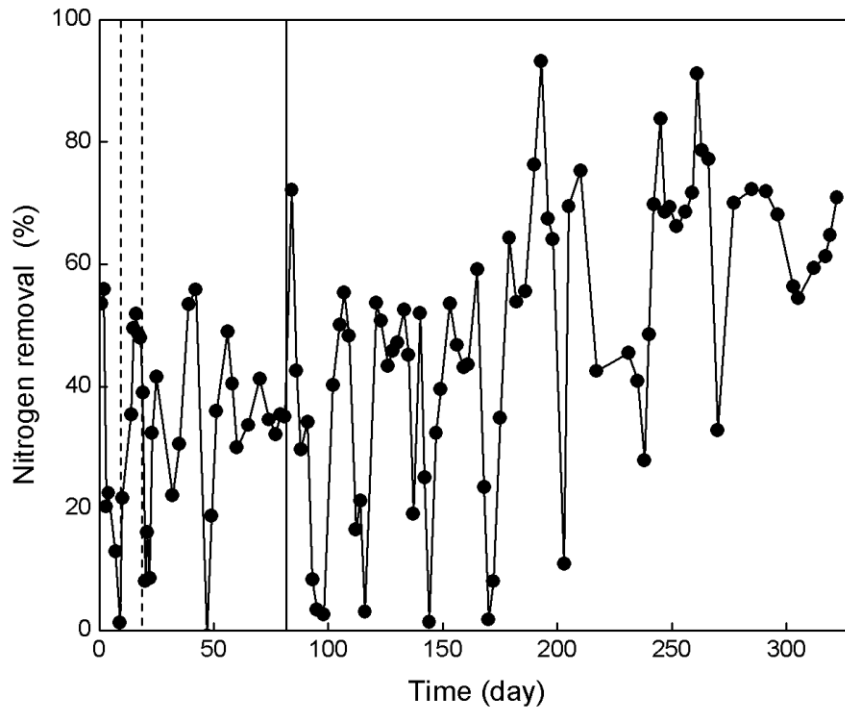


Figure 8 Nitrogen removal of the mainstream MBBR; nitrogen removal = $(\text{Inf. NH}_4^+ + \text{Inf. NO}_2^- + \text{Inf. NO}_3^- - \text{Eff. NH}_4^+ - \text{Eff. NO}_2^- - \text{Eff. NO}_3^-) / (\text{Inf. NH}_4^+ + \text{Inf. NO}_2^-)$

After coupling of two MBBRs, not only ammonium and nitrite were fed into the mainstream MBBR as substrates but also active functional groups were seeded for bioaugmentation. However, given the fluctuation of the sidestream MBBR performance, it was expected to produce additional performance variation in the mainstream MBBR. It was reported that an ammonium/nitrite ratio of 1/1.32 was a preferred rate for an anammox process [97]. Moreover, recently updated stoichiometry suggested the ammonium/nitrite ratio of 1/1.146 [98]. By coupling with the sidestream MBBR, ammonium/nitrite ratio in influent of the mainstream MBBR can hardly be maintained at an optimum level, the optimum condition for anammox reaction, which resulted in the fluctuation of process performance. During day 82 to

day 137, an average nitrogen removal of $36 \pm 20\%$ was achieved, which increased to $45 \pm 23\%$ during the time from day 138 to day 240. Reddish biofilm could be observed from biocarriers at day 322 (Figure 7), implying the successful enrichment of AMX in the biocarrier. Finally, with accumulated AMX in the biofilm biomass, an improved process performance with less fluctuation was accomplished, showed an average nitrogen removal of $68 \pm 12\%$ (Figure 8).

2.3.2 Microbial ecology in coupled sidestream/mainstream system

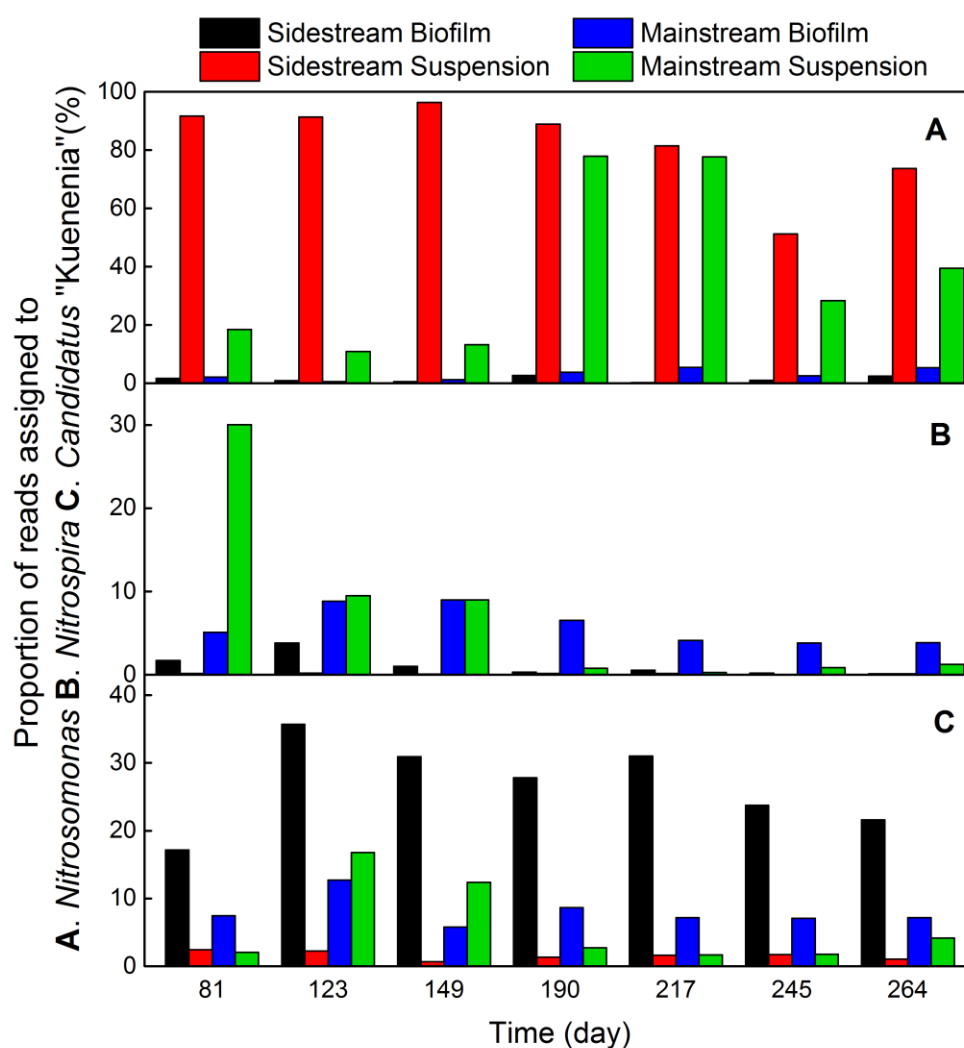


Figure 9 Proportion of reads assigned to A. *Nitrosomonas* B. *Nitrospira* C. *Candidatus* “Kuenenia” at the genus level in suspended and biofilm biomass in the coupled mainstream-sidestream system (based on 16S rRNA gene sequencing)

Illumina platform chemistry was used to identify the overall bacterial community composition. During the whole enrichment period in the MS MBBR process (days), AOB were dominant in sidestream suspension, up to 91.7% reads were assigned to *Nitrosomonas* spp. (Figure 9 A). Fluorescent in situ hybridization (FISH) was applied to explore the distribution of AOB in granule, results showed the extremely high dominance of AOB on the surface of granule [99]. It was also reported that the abundance of *Nitrosomonas* decreased from 2.3% to 0.8% with increasing sludge size from <200 μm to >400 μm [100]. As a one-step anammox reactor, partial nitrification and anammox are happening simultaneously, though AOB prefers to exist in suspension as free cells. As a result, more dominant *Nitrosomonas* was monitored in suspension, while anammox bacteria had a higher fraction of reads assigned to biofilm biomass. Moreover, *Nitrospira* spp. related NOB was extremely low in the ratio of reads assigned in sidestream suspension due to relatively high operating temperature and free ammonia. Results showed enriched NOBs, especially *Nitrospira* spp., in the mainstream biofilm as well (Figure 9 B). It was reported that among three different size of granular sludge (<200 μm , 200 μm - 400 μm and >400 μm), *Nitrospira* spp. had a higher fraction of 4.2% in moderate-sized (200 μm - 400 μm) granular sludge than that in 4.0% small-sized (<200 μm) granular [100]. Other researchers also demonstrated that concentration of *Nitrospira* spp. was enhanced with increasing granular size until 250 μm , after which decrease of fractions were

observed [101]. Also, due to the significantly lower influent ammonium in the mainstream reactor, reduced free ammonium can no longer suppress the growth of *Nitrospira* [102]. Moreover, unlike *Nitrobacter* spp. related NOB, *Nitrospira* spp. related NOB can even survive in complete anaerobic condition; thus, the lower DO in the mainstream reactor had a reduced impact on this genus [25]. Despite reduced DO in the mainstream reactor, results showed no significant change in the presence of aerobic AOB. This can be explained by the flow from the sidestream reactor continuously bringing abundant AOB into the mainstream reactor.

At the end of the enrichment period, the mainstream biofilm carriers showed a stable presence of $7.1 \pm 0.05\%$ reads assigned to *Candidatus* “*Kuenenia stuttgartiensis*” the anammox species used to initially inoculate the sidestream carriers (Figure 9 C). Lastly, as expected due to the low substrates (ammonium and nitrite) concentrations and low temperature, the presence of anammox bacteria in the mainstream reactor remained well below that of the sidestream biofilm (21.6%). However, given the limited amount of AMX transferred from the sidestream suspension (average proportion of $1.6 \pm 0.63\%$ in the suspended biomass of sidestream MBBR), the enrichment of AMX in the biofilm of mainstream MBBR was effective and efficient, achieving a stable presence of $7.1 \pm 0.05\%$ reads assigned to *Candidatus* “*Kuenenia stuttgartiensis*” the anammox species used to initially inoculate the sidestream carriers (9 C). In sum, with no aeration provided in the mainstream MBBR, AMX was more favored than other functional groups. Additionally, instead of suspension, AMX is

more likely to accumulate in biofilm [103], advantage of which was expended by the extra area offered inside the biocarriers.

2.3.3 Efficiency of functional groups in coupled mainstream MBBR

Cell concentrations, substrate consumption rate and molecular tools targeting 16S rRNA, 16S rRNA genes and mRNA could be applied as the indicator for the activity of target bacteria. Method employed in this study was the 16S rRNA amplicon sequencing, which could reveal the activities of all identified bacteria. However, limitations have been reported that 16S rRNA-based molecular technology may not indiscriminately applicable to build the link with physiological activity [104]. In some gram-negative bacteria, such as *Escherichia coli*, the stationary-phase response is indicated by the expression of the *rpoS* gene coding for the σ^S factor [105], but *Nitrosomonas europaea* does not have *rpoS*-like genes completely. Instead, it was demonstrated by multiple researchers that mRNA-based molecular tool is a reliable technology to closely track the activity of targeting bacteria [106-109]. In this study, relative activity was evaluated by apply 16S rRNA sequencing using cDNA (错误!未找到引用源。). In samples from the mainstream biofilm, the percentage of reads assigned to *Nitrosomonas* declined dramatically during enrichment period, which represented the decreasing activity of *Nitrosomonas* (Figure 10 A). Compare to the aeration condition in sidestream anammox reactor, no aeration was provided in mainstream anammox reactor. Although a slight amount of oxygen was brought into the mainstream MBBR through the influent from the sidestream anammox reactor, there was no DO detected within this entire time series experiment. Lack of oxygen

led to the depressed activity of *Nitrosomonas* in the mainstream biofilm. However, percentages of reads assigned to *Nitrosomonas* in mainstream suspension were still dominant (Figure 10 A), which was contributed by constant flow from the sidestream suspension that contained active AOB. As expected, percentages of reads assigned to *Candidatus* “*Kuenenia stuttgartiensis*” in sidestream reactor were relatively high, since the temperature there was optimum. Furthermore, proportions of reads assigned to *Candidatus* “*Kuenenia stuttgartiensis*” in mainstream biofilm increased steadily increasing up to 39.8% (Figure 10 C), showing more enhanced activity, and even the fraction among the whole community remained unchanged. It was reported that the anammox biomass had a lower decrease in activity at lower temperatures after adaption [62]. This is one of the main advantages of enriching the anammox reactor through bioaugmentation. A significant loss of activity would be expected if a strategy was used such as enriching an anammox reactor at high temperature and reducing the operational temperature afterwards, because the fraction of activity loss and time needed for the steady state is uncertain. However, proportions of reads assign to *Nitrospira* spp. were increased from 0.14% up to 9.2% in the mainstream biofilm (Figure 10 B), and were greater in the mainstream suspension than that in the sidestream suspension. With the lack of suppressing from free ammonia and lower temperature, the activity of *Nitrospira* spp. was slightly enhanced in both biofilm and suspension. Overall, without greatly enhancing the activity of *Nitrospira* spp. related NOB, anammox bacteria were not only enriched but also significantly active in the mainstream reactor.

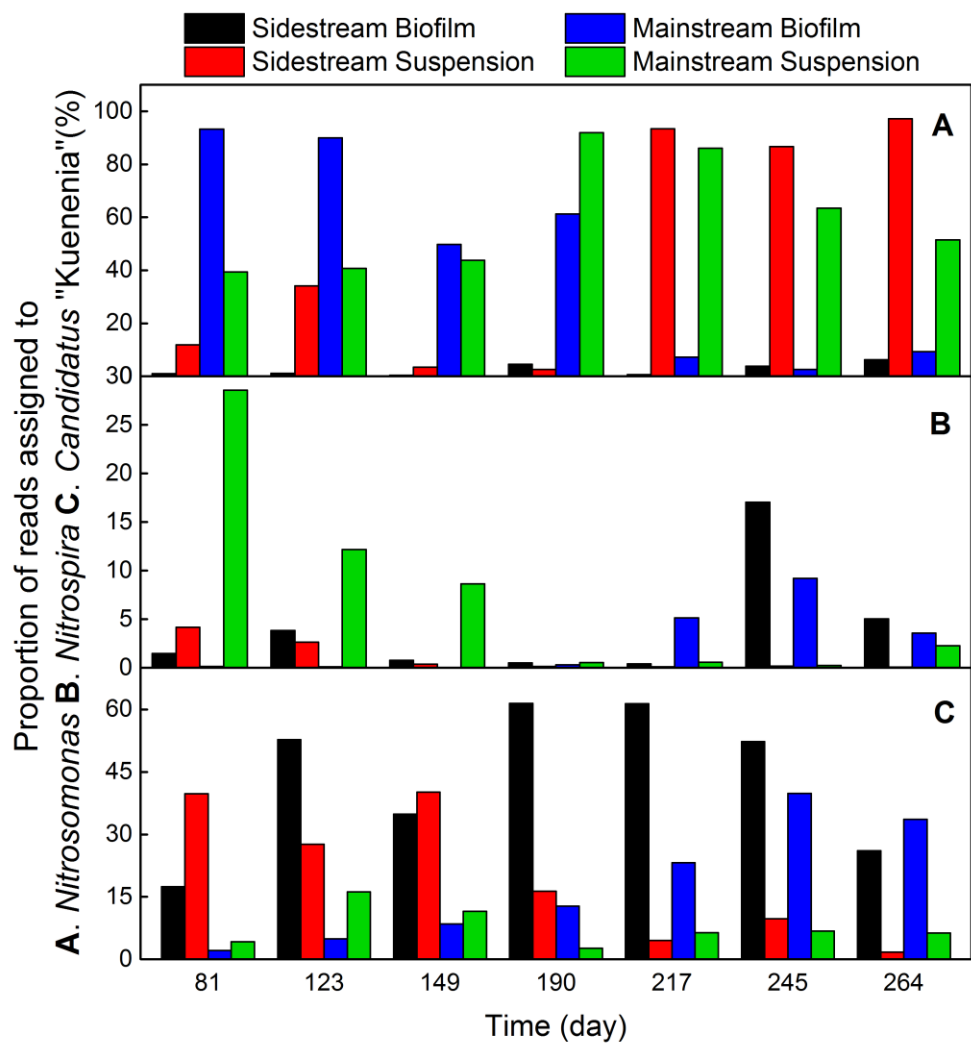


Figure 10 Proportion of reads assigned to A. *Nitrosomonas* B. *Nitrospira* C. *Candidatus* “*Kuenenia*” at the genus level in suspended and biofilm biomass in the coupled mainstream-sidestream system (based on 16S rRNA sequencing)

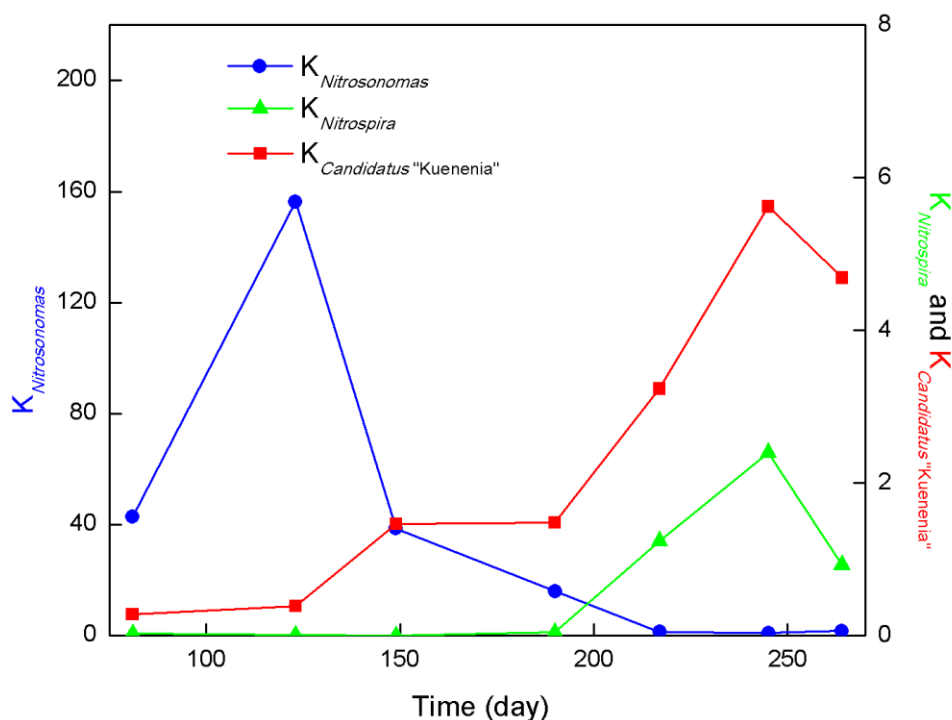


Figure 11 Efficiency of different functional groups in biofilm of mainstream MBBR

2.4 Conclusions

In sum, an anammox MBBR was successfully enriched under low substrates and ambient temperature, achieving an average total nitrogen removal of $68 \pm 12\%$ at the end of enrichment phase. In addition, the coupling of the two MBBRs led to the analogous microbial ecology in the biofilm biomass as well as in the suspended biomass, which included the accumulated *Nitrospira* spp. related NOB. However, it needs to be emphasized that, even with the limited fraction of *Candidatus* “*Kuenenia stuttgartiensis*” in the coming suspension from the sidestream MBBR, *Candidatus* “*Kuenenia stuttgartiensis*” was still effectively enriched in the biofilm biomass. Moreover, results from sequencing analysis demonstrated the rising activity of

Candidatus “*Kuenenia stuttgartiensis*” through this whole time series experiments, and achieved the most competitive level among all functional groups. These results are critical to address the significance and advantages of bioaugmentation in the enrichment of the mainstream anammox-based biological nitrogen removal (BNR) processes.

Chapter 3. Enrichment and Characterization of an Anammox Moving Bed Biofilm Reactor (MBBR) to Treat Mainstream Wastewaters at Low temperature

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3.1 Introduction:

Anammox bacteria (AMX) were first detected in Delft University around 20 years ago [110]. Owing to the unique property to oxidize ammonium in the absence of oxygen and organic carbon, anammox bacteria and the related technologies earned their critical position in the nitrogen removal process from real wastewater treatment plants (WWTPs). In WWTPs conventional nitrogen removal is accomplished in two steps: nitrification, carried out by AOB and NOB, denitrification carried out by denitrifiers, which reduce nitrate to dinitrogen gas with organic carbon as the electron donor. Aeration supplied to the nitrification and organic substrates utilized in denitrification was studied, and it was demonstrated that the conventional nitrogen removal process is remarkably energy consuming and associated with the excess production of sludge. However, by fully applying anammox process in WWTPs, a 60% reduction in the oxygen demand could be achieved [111], that is approximately 50% of the electricity consumption in WWTP [91].

Considering the significant economic potential of the anammox process in WWTPs, the first full-scale application was implemented successfully in the sidestream in Rotterdam (NL) [112]. Since then, applications using the anammox process have been installed in the sidestream of many WWTPs to treat the ammonium-rich wastewater, such as the Dokhaven WWTP in the Netherlands [113], Blue Plains WWTP in Washington, DC [114]; Strass WWTP in Australia [115]; Water Reclamation Plant in Changi, Singapore [116] and Gaobeidian WWTP in Beijing, China [117]. According to

the reported data, more than 200 full-scale plants using anammox technologies have been operated in 2014 [111].

Although the anammox technologies have become increasingly mature in the sidestream, only 15%-20% of nitrogen loadings are present in the sidestream processing, while the bulk of it still needs to be removed from the mainstream[118]. Research interests thus have shifted from sidestream to mainstream applications. One of the main challenges of mainstream application is to reduce the operational temperature from over 30 °C in the sidestream to below 20 °C in the mainstream [119], which, however, could lead to impaired biomass specific removal rate [120]. Enrichment of desired biomass proves an effective way to mitigate the effect of changing operational conditions [121, 122]. The anammox activity reached 30-44 mg-N/gVSS/d at 10°C for enriched anammox biomass [123] and a high value of 42 g-N/gVSS/d was achieved at 10 °C for enriched suspension biomass [124]. Bioaugmentation methods like selective recycling by cyclone [115], screens [125], segregation in cyclic aerobic/anaerobic condition [126] and sieving with specific mesh size [114] were applied to selectively enrich high AMX ratio activated sludge.

To date, most studies focus on the impacts of mainstream operational conditions to already enriched anammox reactors, which were further enriched by inoculating massive amount of AMX biomass [118, 124, 127-129]. Directly enrichment of bacteria within the low temperature conditions especially for an MBBR, on the other hand, lacks sufficient research. Furthermore, many researchers point toward the issues related to post-treatment of the sidestream effluent regarding the residual suspended

solids, ammonium, nitrate and nitrite [124, 130, 131] [132] [128, 133], which needs further exploration.

Thus, this study suggested a novel coupling strategy to directly seed mainstream reactor with the effluent of a sidestream reactor, which allows the post polishing and wasted sludge utilization of sidestream effluent. The overall objective of this study was to characterize the seeded microbial community in the mainstream reactor through performance measurements and molecular genetic sequencing. The sub-goal was to evaluate the impact of coupling of the two reactors.

3.2 Materials and methods

3.2.1 Experimental set-up and reactor operation

In this study, two 6 L lab-scale moving bed biofilm reactors were operated for over 500 days at 15 ± 2 °C and 35 ± 1 °C respectively. Effluent from sidestream MBBR was continuously fed into mainstream MBBR as the inoculum by coupling of the two reactors. Both reactors were filled with 2000 Kaldnes 1 biocarriers, which occupied 33% of the total reactor volume. The same influent flow rate of 4 L/d was used resulting in an HRT of 1.5 days. DO levels were monitored at 0.17-0.68 mg O₂/L, while the mainstream reactor was operated under anaerobic conditions throughout the whole time series experiments.

Fed with only ammonium using (NH₄)₂SO₄ stock solutions, the sidestream influent concentration went through variations between 406 mg NH₄⁺-N/L and 886 mg NH₄⁺-N/L as a means of optimizing the effluent quality according to the reactor

performance. The mainstream reactor was fed with the effluent from sidestream which both contained the remaining ammonium (100 ± 3.6 mg NH_4^+ -N/L on average) and nitrite (31 ± 1.1 mg NO_2^- -N/L on average). 1M NaHCO_3 was prepared to maintain the pH range at 7.45-7.55 except for the period from June 23rd to June 30th, 2017, during which the pH increased to 8 for a week.

3.2.2 Performance tests

The substrate concentration in both influent and effluent was measured three times a week with duplicates. Ammonium, nitrite and COD concentrations were determined spectrometrically using high-range (0-50mg NH_4^+ -N/L) AmVer™ Salicylate Test 'N Tube (Hach Chemical Co., Loveland, CO), Ricca Color reagent (Ricca chemical company, Alington, TX. Cat#2233.5-32) and high range (20-1500 mgCOD/L) COD Digestion vials (Hach Chemical CO., Loveland, CO) respectively. Additionally, nitrate concentration measurements were performed using Ion chromatography (Thermo Scientific Dionex ICS-2100., Sunny-vale, CA) after filtering through 0.2 μm syringe filters (A chemteck Inc. Cat# 0503-000007). A real time monitor of DO was developed using Clark-type electrodes (YSI 5331, Yellow Springs, OH) which interfaced to an online DO meter (YSI 5300A, Yellow Springs, OH) [134] and pH was measured by pH/ORP controller (JENCO. Electronics, LTD).

3.2.3 Biomass collection and DNA extraction

Biomass was withdrawn for both suspension and biofilm once a week from the two reactors. RNA Protector (Qiagen, Germany Lot # 154044773) was added for DNA

protection and all the samples were stored in a -80 °C refrigerator before further microbial analysis. Genomic DNA was extracted using DNeasy mini kit (Qiagen, Inc., Germantown, MD) following the user manual. According to the performance curve of Qiacube (Qiagen, CA), a diluted or combined sample of total cell numbers of approximate 2×10^9 as required for optimal performance. The resulting DNAs were quantified using dsDNA HS assay on the UV NanoDrop Lite spectrophotometer (Thermo Scientific, Madison, WI). In case the ratio of A260/A280 was higher than 1.8, a quantitative PCR analysis was conducted to verify the purification of the resulting DNA samples. Pla45f (5 µm) and Amx667r (5 µm) were selected for Anammox amplification running with the set-up protocol for 45 minutes by C100 Touch Thermal Cycler and CFX 384 Real-Time system (Bio-rad). Qualified DNA was stored at -20 °C prior to sequencing preparation.

3.2.4 16S rRNA gene sequencing and post-analyzing

A set of 24 samples were selected from two reactors for both biofilm and suspended biomass. V8 hypervariable region of the 16s rRNA gene was targeted to analyze all the diverse groups within the bacterial communities. Barcoded Fusion primers with sequencing adaptors were employed to amplify the extracted DNA and prepared for the equal-molar DNA library. Qualification and quantification of products equaled 400 bp base length was obtained by using an Agilent Bioanalyzer 2100 instrument. After dilution to equimolar pool, the library combined with amplification solutions were loaded on Ion OneTouch2 instruments to prepare template-positive ISPs, which went through further enrichment on an Ion OneTouchTM ES instrument using Ion

PGM Hi-Q View OT2 Kit. Final sequencing was procured by using the Ion 318 Chip v2BC installed on Ion Torrent Personal Genome Machine™ (PGM) (Thermo Fisher, MA) which performed the sequencing following the manufacturer's published protocols.

All the sequencing datasets were post analyzed using mothur (version 1.34.3). Raw data was treated for the elimination of reads with short length (<300 bp), poor end quality (<20), high homopolymeric region (>8bp) and chimeras. The references for alignment and classification were chosen as Silva ribosomal database nr ver. 128.

3.2.5 Calculation of nitrogen removal efficiency

The definition of the nitrogen removal efficiency is the removal of total nitrogen including ammonia, nitrite and nitrate. The calculation was conducted by dividing the difference of total nitrogen concentration between influent and effluent by the influent nitrogen source concentration. Since the mainstream reactor was operated under the anoxic condition where only anammox bacterial were expected to survive, the calculation of influent nitrogen source in mainstream should be based on the basic stoichiometry equation of the anammox process [135] and neglect the effect of remaining nitrate from the sidestream. The calculation equations are as follows:

Sidestream Nitrogen removal efficiency

$$= \frac{(\text{Influent } [\text{NH}_4^+] - \text{Effluent } [\text{NH}_4^+] - \text{Effluent } [\text{NO}_2^-] - \text{Effluent } [\text{NO}_3^-])}{\text{Influent } [\text{NH}_4^+]}$$

Mainstream Nitrogen removal efficiency

$$= \frac{(\text{Influent } [\text{NH}_4^+] + \text{Influent } [\text{NO}_2^-] + \text{Influent } [\text{NO}_3^-] - \text{Effluent } [\text{NH}_4^+] - \text{Effluent } [\text{NO}_2^-] - \text{Effluent } [\text{NO}_3^-])}{\text{Influent } [\text{NH}_4^+] + \text{Influent } [\text{NO}_2^-]}$$

3.3 Results and discussion

3.3.1 Reactors performance and the major affected parameters

Operated for almost 500 days, the two MBBRs were coupled through whole time series experiments to utilize the biomass from sidestream effluent and directly enrich the biofilm biomass under low temperature in the mainstream reactor. As Figure 12 shows, the whole operation process of the sidestream reactor could be divided into four phases according to the influent ammonium concentration and the total nitrogen removal efficiency.

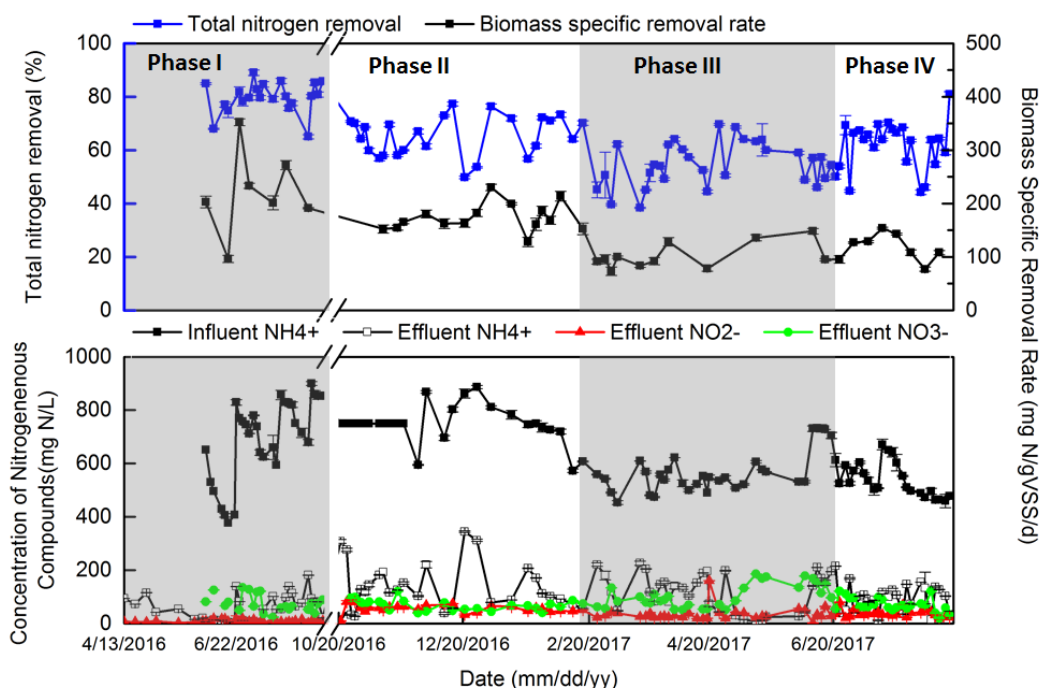


Figure 12 Sidestream performance from Apr. 2016 to Aug. 2017; results represent average and standard deviation of duplicate experiments.

The sidestream MBBR performed a total nitrogen removal of $64 \pm 1.8\%$ on average. In Phase I, the influent ammonium was $724 \pm 122 \text{ g NH}_4^+\text{-N/L}$, which resulted in a relatively low effluent nitrite ($<5 \text{ mg-N/L}$) and ammonium concentration ($<100 \text{ mg-N/L}$), therefore an average total nitrogen removal of $77 \pm 2.0 \%$ was achieved. However, a decreased average total nitrogen removal of $65 \pm 1.2\%$ occurred in Phase II, which was evidenced by the rising ammonium and nitrite concentration in the effluent. This was likely caused by the biomass decay and the presence of extracellular polymeric substances (EPS) [136, 137] which occupied the area and space for biomass to grow and blocked the contact between substrate and anammox bacteria. Consequently, the influent ammonium concentration declined to $542 \pm 42 \text{ NH}_4^+\text{-N/L}$ in Phase III, which aimed to recover the performance and optimized the effluent qualities. However, the reduced ammonium concentration together with lower free ammonia (FA) concentration led to a nitrate accumulation up to $156 \pm 22 \text{ mg NO}_3^-\text{-N/L}$ which further lowered the nitrogen removal efficiency to only $54 \pm 0.93\%$. Thus, an elevated influent ammonium concentration of $706 \pm 43 \text{ mg NH}_4^+\text{-N/L}$ as applied at the end of Phase III, which resulted in a significant decrease of nitrate concentration down to $75 \pm 25 \text{ mg NO}_3^-\text{-N/L}$ in Phase IV. Nitrite accumulation up to $186 \text{ mg NO}_2^-\text{-N/L}$ was also observed during Phase III, which might exert a negative impact on anammox bacteria activities as well as the total nitrogen removal.

Previously, a 75% reduction in anammox activity due to nitrite accumulation has been reported in a highly enriched anammox system operated at 10°C [138]. Notwithstanding, the sidestream reactor only showed a short time nitrite accumulation

and recovered quickly in this study, which was similar with other results at 15 °C [127, 139] or lower at 10-12 °C [118].

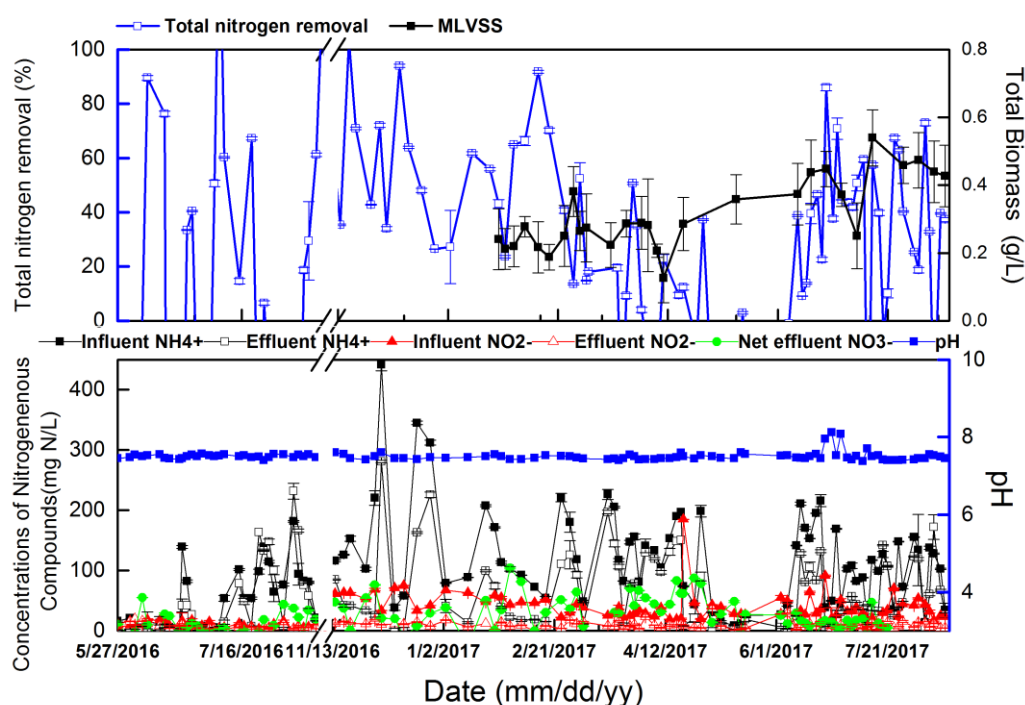


Figure 13 Mainstream in-situ performance from May 2016 to Aug. 2017; results represent average and standard deviation of duplicate experiments.

The performance of mainstream reactor was illustrated in Figure 13. The total biomass amount was calculated based on COD measurements and converted to MLVSS thereafter. A significant increase of MLVSS (including both biofilm and suspension biomass) from 0.25 ± 0.050 g/L to 0.46 ± 0.040 g/L was observed 6 months after initialization of mainstream reactor. Despite that, the suspension biomass concentration highly relied on the biomass concentration of the sidestream, the growth of biomass in biofilms from 0.33 ± 0.080 g-COD/carrier to 0.80 ± 0.050 g-COD/carrier indicates a successful enrichment of the mainstream reactor. A total

biomass specific activity of 119 ± 30 mg-N/gVSS/d was achieved, where anammox specific activity down to 10 g-N/kgVSS/d [128] was observed in MBBR operated at 10 °C as well as a value <20 mg-N/gVSS/d [127] at 15 °C. The high specific removal rate was likely attributed the dominant fraction of AMX related bacteria (*Candidatus "Kuenenia"*) on the mainstream biofilm ($>50\%$).

A delay in the change of nitrate concentration between sidestream and mainstream was monitored due to a high HRT of 1.5 days (compared to regular HRT of 6-7 h in WWTPs), which resulted in an increased nitrogen removal percentage to above 100%, or a decrease below 0%, shortly. Despite these errors, the mainstream total nitrogen removal, which showed a growing removal proportion from $39 \pm 11\%$ (11/14/16-6/23/17) to $47.00 \pm 5.4\%$ (6/23/17-8/16/17), has finally reached an average total nitrogen removal of $42.16 \pm 4.92\%$. This result was lower than the average total nitrogen removal of the sidestream as expected (which proved that the negative effect of low temperature on the activity of both AOB, NOB and anammox bacteria [140] was not neglected, it was comparable to the value of around 40% [125] in the one-stage partial nitrification/anammox reactor operated at 15 °C while still lower than the value over 70% [141] in an 12 L MBBR reactor for partial nitrification/anammox process under 15 °C. When considering the major affected parameters to the total nitrogen removal efficiency, the sidestream MBBR was largely affected by the overgrowth of EPS, while the fluctuation of the mainstream MBBR performance was influenced by a combined effect of both the sidestream effluent quality and operational conditions of the mainstream MBBR.

Although a decreased sidestream influent ammonium concentration down to 550 mg NH_4^+ -N/L successfully optimized the effluent ammonium (influent ammonium for mainstream) from 146 ± 3.2 mg NH_4^+ -N/L to 23 ± 1.5 mg NH_4^+ -N/L, which was more suitable for a mainstream anammox process. Less supplied ammonium in the mainstream reactor was possibly insufficient for AMX to convert all nitrite into nitrogen gas; thus it had a higher concentration of residual nitrite, which could suppress AMX activities. In the meantime, an elevated number of NOB-related bacteria (*Nitrospira* spp.: from 2.1% to 6.6% in biofilm and 3.7% to 10.3% in suspension) was also monitored in the sidestream MBBR. Thus, more NOB were inoculated into the mainstream MBBR, which resulted in a net nitrate production from 20 ± 0.60 mg NO_3^- -N/L to above 62 ± 0.59 mg NO_3^- -N/L during May 2017.

Taking into account of the negative impacts, inhibition of NOB activity was essential under lower ammonium concentration. Based on kinetic parameters of AOB, NOB and AMX, many methods were applied in past studies for successful suppressions such as real-time DO control[31, 115, 116, 141, 142], intermittent aeration[129, 143-146], chemical compounds addition[147] and pH adjusting [142, 146, 148].

DO control is defined by many as the key method for suppressing NOB. Theoretically, AOB have a higher affinity to oxygen than NOB, meaning that limiting DO levels in the range of 0.15-0.18 mg- O_2 /L allows for stable suppression of NOB activity at 15 °C [141]. However, prior researches have indicated that a relatively high DO of around 1.5 mg- O_2 /L was also beneficial to mainstream nitrification since the DO half saturated value of AOB was sometimes higher than that of NOB [115, 142]. Intermittent

aeration [143, 144] with alternating aerobic and anoxic phases was also designed to select AOB against NOB in SBR. The effect of intermittent aeration was probably due to the lag time for enzymes in NOB to respond [129, 145] or a nitrite limitation condition [146]. Nevertheless, some researches demonstrated that intermittent aeration did not constitute a sufficient way to suppress the NOB, especially for the protected NOB inside a biofilm reactor [129]. Considering the different operational conditions like the floc size or biofilm thickness, the ideal DO concentration varies for a successful out-selection of NOB [31].

In this study, a low DO level at 0.17-0.68 mg-O₂/L was applied in the sidestream, along with high ammonium concentration, and achieved a successful repression of *Nitrospira* spp. in the sidestream MBBR, yet more efficient methods were needed to maintain the low proportion of NOB under the low ammonium concentration condition. Moreover, with no aeration provided in the mainstream MBBR, the limited oxygen consumed was only from the residual DO in the effluent of the sidestream MBBR, which maintained a stable DO of 0 mg-O₂/L in the mainstream MBBR. In addition, the rising ammonium concentration along with a short period pH increase to above 8 at the beginning of July resulted in an effective suppression of *Nitrospira* spp. related NOB from 8.9% to 2.2% in the mainstream biofilm together with a net nitrate production reduced to below 20 mg NO₃⁻-N/L. This corresponds to other researches that the higher pH value together with an increased ammonium concentration could have a FA (free ammonia) accumulation, thus effectively improving the NOB out-selection especially for *Nitrospira* spp. [114, 148, 149].

3.3.2 Microbial community and diversity

Both the suspension and biofilm samples of sidestream and mainstream were analyzed using 16S rRNA gene sequencing from February to August 2017. The results from two reactors demonstrated an analogous spatial distribution trend of microbial ecology (Figure 14); namely that the biofilm biomass was dominated by AMX while AOB were detected mostly in suspended biomass. This could be explained by the insufficient oxygen transportation inside the biofilms, which limited the growth of AOB but favored the growth of AMX in the opposite [128, 150, 151].

Both AOB and NOB related bacteria were detected in the mainstream reactor though no excess aeration was supplied. The remaining dissolved oxygen from sidestream effluent could possibly provide the growth conditions for these two aerobic bacteria. AMX related bacteria (*Candidatus "Kuenenia"*) represented about 30% of the biofilm communities in sidestream while a sharp increased fraction from 12% in February 2017 to above 60% in March 2017 could be observed in mainstream biofilm (Figure 14). An average fraction of $46 \pm 6.5\%$ could be reached and stabilized in the following 4 months from April to August 2017. Meanwhile, a relatively stable proportion of *Nitrosomonas* in suspended biomass was demonstrated both in sidestream ($60 \pm 4.4\%$) and mainstream ($54 \pm 9.4\%$), which was mostly contributed by the bioaugmentation (Figure 14).

The genus level microbial diversity showed high identity between the sidestream and the mainstream MBBRs, where the major group ($> 99.9\%$) of AMX, AOB and NOB were related to *Candidatus "Kuenenia"*, *Nitrospira* and *Nitrosomonas*, respectively.

Compared to *Nitrospira*, *Nitrobacter*-related NOB, which was only presented in suspension, occupied a very limited amount ($< 0.01\%$). This could be ascribed to a lower nitrite half-saturation constant (0.52 ± 0.14 mg-N/L) and a higher yield (0.14 ± 0.02 mg-COD/mg-N) of *Nitrospira*, which benefited their growth under low nitrite mainstream conditions [25]. The overall analogy of microbial communities between the two reactors was owing to the coupling. The overwhelming fraction of enriched populations related to *Candidatus "Kuenenia"* (20-30%) into the mainstream MBBR together with a referable feeding conditions, fostered a more resilient and competitive microbial community after changing operational conditions (decreased temperature and substrate concentration). Although the coupling of the two reactors imposed a significant impact on the microbial ecology in the mainstream MBBR, other studies without coupling also showed no change in the predominant bacterial community composition at genus level due to the decreased temperature [121, 122, 141].

In this study, with the next-generation sequencing technologies, the growth of *Chlorobi* like SJA-28 and *Chloroflexi* like SBR2076 were revealed together with anammox bacteria (Figure 15). Although the *Chlorobi* related bacteria did not compete with AOB or AMX for energy or carbon resource, they still exerted a negative influence on the performance of the coupled system by occupying growth space on biocarrier. However, since this bacteria is photoautotrophic bacteria, the cover of the mainstream MBBR by aluminum foil could prevent the light thus build a limitation on further accumulation. Other microbial community like *Denitratisoma*, which might cause intermediate accumulation of nitrite during the reducing of nitrate

[152], only constituted of a fraction below 4% (Figure 15). Thus the contribution to the nitrite accumulation could be neglected during the experiment.

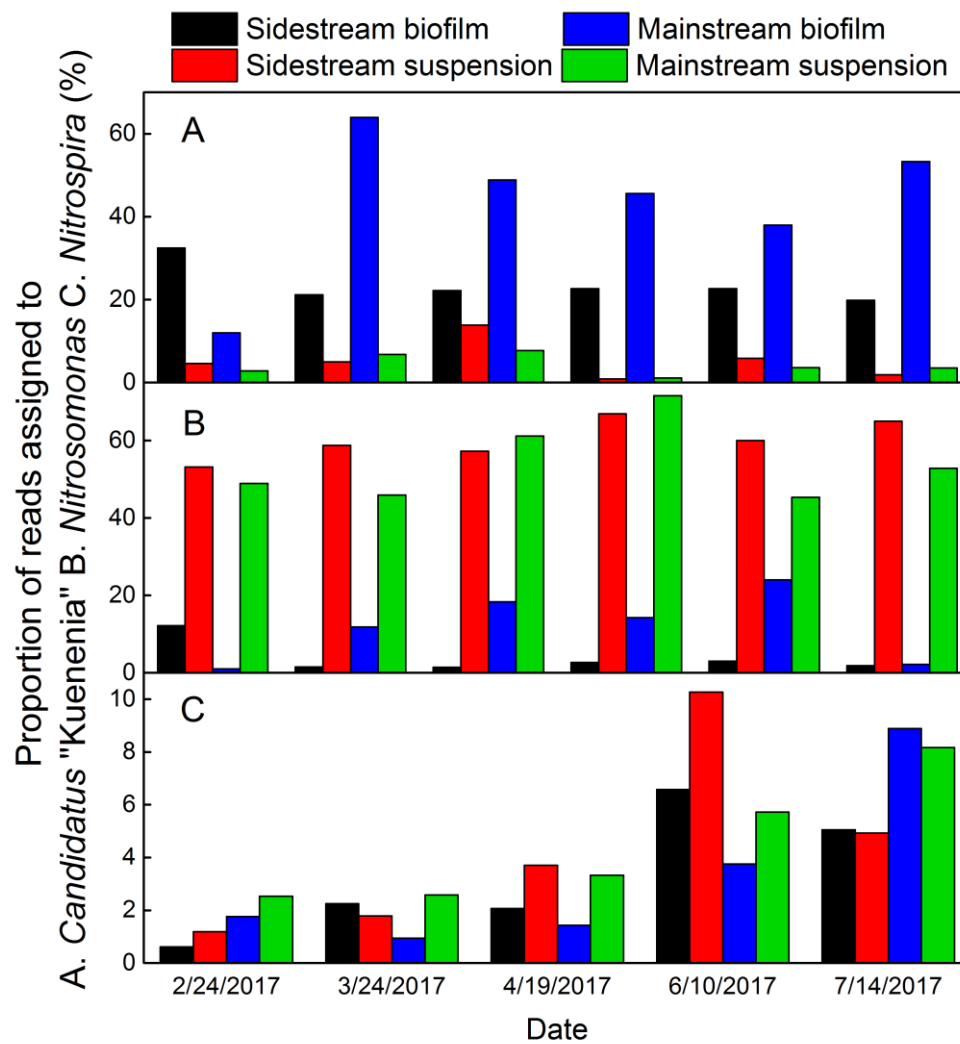


Figure 14 Proportion of reads assigned to A. *Candidatus* "Kuenenia" B. *Nitrosomonas* C. *Nitrospira* in both biofilm and suspended biomass of the coupled mainstream-sidestream system (based on 16S rRNA gene sequencing)

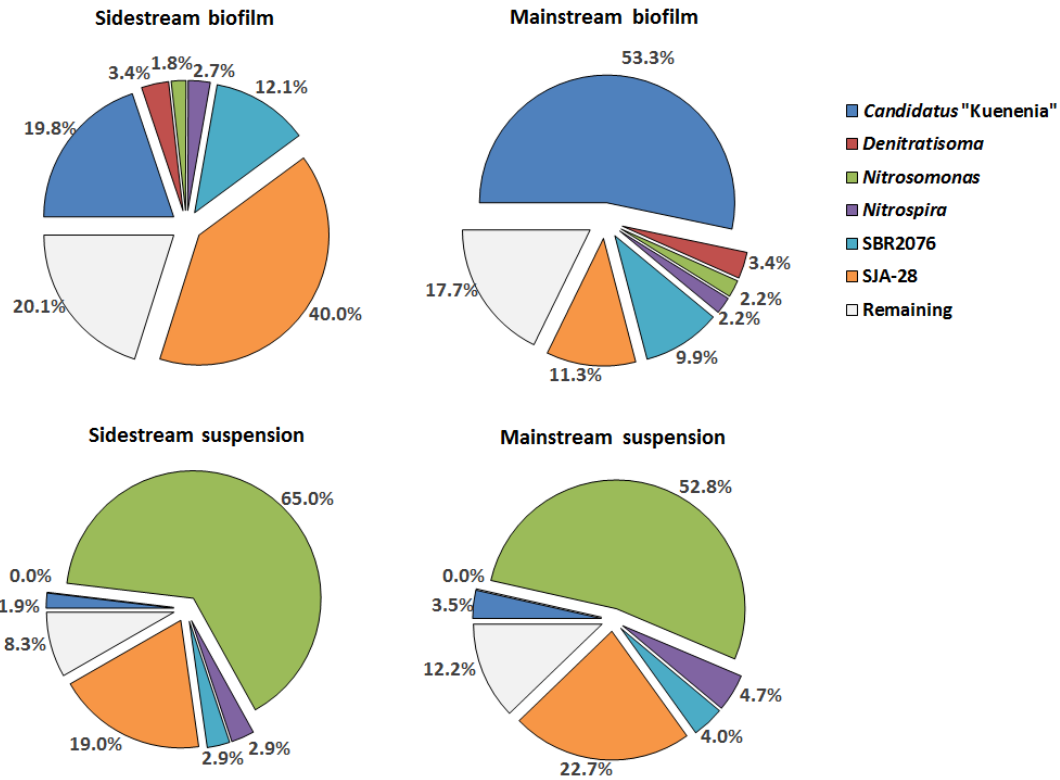


Figure 15 Microbial community composition of biofilm and suspended biomass of the sidestream and mainstream MBBR on 8/25/2017. Pie slices with fractional percentages represent relative abundance of each dominant genera as well as the “remaining” populations.

3.3.3 Evaluation of the reactor coupling

Based on the design purpose, the connection of sidestream to mainstream would allow for a better reuse and polish of sidestream effluent, which reduced the cost of buying biomass-filled carriers and selected for potential competitive species within the directly enriched biomass. According to the experimental results, although no specific bacteria were selected through this operating mode, a successful enrichment was evidenced with an extremely high fraction of anammox bacteria(> 50%) thus a relatively higher biomass specific removal rate of $119 \pm 30 \text{ mg-N/gMLVSS/}$ was

achieved compared to other reactors operated under low temperatures (10 g-N/kg/VSS/d at 10 °C period [128]; below 20 mg-N/gVSS/d at less than 15 °C [127]; 30–44 mg-N/gVSS/d at 10 °C or enriched anammox biomass [123]). These results suggested a promising potential for such enrichment strategy. However, due to the unstable performance of effluent from sidestream, the mainstream was highly affected by this connecting.

3.4 Conclusions

In this study, two MBBRs operated under sidestream and mainstream temperature conditions were coupled to directly enrich the biofilm biomass under low temperature by continuously feeding the mainstream MBBR with effluent from the sidestream MBBR. The main conclusions are summarized as follows:

- (1) Successful enrichment with the extremely high fraction of anammox bacteria (> 50%) was achieved in the mainstream MBBR showing an increasing biofilm biomass from 0.33 ± 0.08 g-COD/carrier to 0.80 ± 0.050 g-COD/carrier. Relatively high biomass removal rate of 119 ± 30 mg-N/g-MLVSS/d was also demonstrated.
- (2) An effective inhibition of *Nitrospira* spp. was demonstrated by increasing the influent ammonium concentration together with a short time pH change to 8. Both reactors recovered quickly from a short nitrite accumulation.
- (3) The microbial diversity did not show significant changes between the two reactors with both the *Candidatus* "Kuenenia", *Nitrosomonas* and *Nitrospira*

related bacteria (> 99.9%) represented as the dominant AMX, AOB and NOB genera. Similarly, the geometry of the distribution had no difference between side and mainstream reactors.

- (4) The presence of *Chlorobi* and *Chloroflexi* was commonly reported to co-exist in the anammox system, it might be partly contributed by the exposure of the system to light and further research to elucidate the mechanism and inhibition of *Chlorobi* are required.

Consequently, successful enrichment has been achieved in the mainstream reactor where the COD of collected biomass from biofilm was doubled as well as a rising fraction of anammox related bacteria *Candidatus "Kuenenia"* from 12% to above 50%. A total nitrogen removal efficiency of $42 \pm 5.0\%$ was also estimated for the mainstream reactor. Combining the qPCR and next-generation sequencing technologies, more information about the whole microbial community diversity was revealed among the different reactors and during different periods of experiment times.

Chapter 4. Effect of Organic Matter on the Performance and Microbial Ecology of a Mainstream Anammox Process

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4.1 Introduction

Anaerobic ammonium oxidation (Anammox) process is an environmental friendly, cost-effective biological nitrogen removal (BNR) process. Compared with a conventional BNR process, anammox can reduce energy consumption for aeration and no organic carbon is needed [32-34]. Actually, instead of conventional activated sludge processes designed for full biological nitrogen removal by nitrification and denitrification, the organic matter contained in raw influent could be concentrated and collected, then further transformed into methane through anaerobic digestion.

To date, the anammox process has been implemented successfully in over 100 full-scale plants by now [7]. Among all these applications, most of them were implemented with high strength ammonia wastewater treatment in sidestream in municipal WWTPs [7]. Nonetheless, as sidestream only accounts for around 20% of total nitrogen loading in WWTPs, more attention has been attracted to the implementation of anammox process in mainstream waters.

There are several challenges in implementing anammox under mainstream conditions. The first challenge is the low activity of anammox bacteria at low temperature ($< 15^{\circ}$) and variation of temperature in different seasons ($10 - 25^{\circ}\text{C}$) [42] compared to the high and stable temperature ($>30^{\circ}\text{C}$) in the sidestream process [42, 153]. The specific activity of anammox bacteria can drop dramatically when the temperature went down from 30 to 10°C [154]. Even at 30°C , maximum specific growth rate of anammox bacteria ($0.05\text{-}0.09/\text{day}$) [39, 56] is extremely lower than that of AOB ($0.7\text{-}0.9/\text{day}$) [155, 156]. These differences in activities and growth rates under various

temperatures can lead to a shift in microbial ecology [154, 157, 158], and it's difficult to retain enough anammox bacteria, which is critical for mainstream anammox under low temperature conditions.

Besides low temperatures, for low strength municipal sewage in the mainstream process, reliable autotrophic nitrogen removal under high COD/N has not been well developed [69]. Based on different applied treatment processes, COD/N ratio in the influent of anammox process could vary between 1/1 and 10/1 [40, 81, 82]. Although, it is believed that the anammox process has better performance when the biodegradable COD/N ratio is lower than 0.5 [159], there also was a successful study that showed increased nitrogen removal efficiency up to 95%, when the COD/N ratio increased from 0.5 to 1.7 [85]. Considering the 95% removal efficiency cannot be achieved only by using the anammox process, a system with coexistence of both the anammox process and denitrification might have the potential of better nitrogen removal ratio. Generally, it is believed that denitrification is more favorable than the anammox process since AMX has a longer doubling time and is highly sensitive to temperature fluctuate [98, 160]. Although the coexistence of denitrifying bacteria and AMX has been reported to be beneficial to granulation of AMX due to the competition for nitrite [161], link between co-benefit on granulation and competition for nitrite is not clear. Other studies showed that AMX would lose activity when the COD/N was above 1.0 [75]. Moreover, it was reported that *Candidatus* "Anammoxoglobus propionicus" could out compete other anammox bacteria and heterotrophic denitrifiers when propionate was introduced as the supplementary

electron donor in addition to ammonium [79]. It was also shown that *Candidatus* “*Brocadia fulgida*” could out compete other anammox bacteria in the presence of acetate in continuous cultivation experiments[80]. To date, most studies focus on the impacts of imposing organic carbon on anammox reactors under sidestream operational conditions. Here we report the rarely studied impacts of imposing organic carbon to an anammox moving-bed biofilm reactor (MBBR) newly enriched under mainstream influent conditions.

The principal goal of this study was to determine the impact of additional organic carbon on the microbial ecology and performance of a mainstream anammox MBBR. We hypothesized that anammox bacteria have the ability to assimilate organic carbon under certain condition. The specific objectives were to: 1) investigate the shifts of the microbial community in both biofilm and suspended biomass in response to the imposition of and recovery from organic carbon dosing, 2) explore the catabolism and anabolism of the functional groups in the mainstream MBBR with, and without, the exposure to the additional organic carbon.

4.2 Materials and methods

4.2.1 Reactor configuration and operation during additional organic carbon phase



Figure 16 Mainstream MBBR during additional organic carbon phase

The impacts of organic carbon were studied using a 6 L mainstream MBBR enriched as described in Chapter 2, which contained 2000 Kaldnes K1 biocarriers with a total volumetric fill capacity of 33%. The reactor was initially

coupled with a 6 L sidestream anammox MBBR, and was uncoupled after being successfully enriched. The mainstream MBBR was operated at 24.5°C with a hydraulic retention time (HRT) of 1.5 days and was fed with 33 ± 1.3 mg-N/L ammonium and 32 ± 0.74 mg-N/L nitrite after being uncoupled. Organic carbon (Table 2) was dosed into the mainstream MBBR through the influent after 14 days, dosing lasted for 28 days. Composition of organic carbon presented in real WWTP varies according to the different source of influent and normally contain various types, including readily biodegradable COD (RBCOD), slowly biodegradable COD (SBCOD) and inert [162]. Furthermore, the application of mainstream anammox is more feasible for the wastewater waters processed with primary treatment to partly reduce the COD/N ratio [163, 164]. Hence the recipe of synthesized organic carbon employed in this study included RBCOD, SBCOD and inert, to result in a total concentration of 200 mg-COD/L (Table 2). After organic carbon dosing was stopped, the reactor was further monitored for 56 days.

Table 2 Recipe of organic carbon

		Conc. mg COD/L
Sodium acetate		50.0
Milk powder (150mg COD/L)	Lactose (50%)	75.0
	Protein (35%)	52.5
	Ashe (10%)	15.0

	Other (5%)	7.5
Total COD		200.0

4.2.2 Anammox and denitrification batch activity tests

During imposing organic carbon into the mainstream MBBR experiment, batch tests were implemented to monitor the activity of anammox and denitrification before (day 14), during (day 28, 42) and after (day 56, 77, 96) dosing organic carbon. For each batch test, 10 carriers were removed from the mainstream MBBR and washed three times with distilled water to remove any chemical residue. Along with oxygen free media, 10 carriers were stirred in a 150-mL conical flask until substrates (ammonium and nitrite) were consumed. Three types of batch tests were conducted to understand the kinetics of anammox process (Test A), anammox process with presence of organic carbon (Test B) and denitrification process (Test C) (Table 3). Each of these batch tests was conducted in duplicate.

Table 3 Substrates added in batch tests

	ammonium mg-N/L	Nitrite mg-N/L	Nitrate mg-N/L	Acetate mg COD/L
Test A	30	30	N/A	N/A
Test B	30	30	N/A	50
Test C	N/A	N/A	10	50

4.2.3 Sample collection and DNA extraction

For lab-scale reactors, biomass samples from suspension and biofilm were collected each sampling day and stored at -80 °C for subsequent molecular processing. For suspension biomass sampling, a 30 mL suspension from each reactor was taken and transferred into a 50 mL sterile tube (Corning, MA) with 50 mL sterile pipet. The sample was centrifuged after centrifuge (Beckman Coulter, NJ) at 4 °C and 8000 G for 10 minutes, the supernatant was discarded and biomass was resuspended in 1 mL RNA protect reagent (Qiagen, CA). For biofilm biomass sampling, one carrier was sacrificed in each reactor and replaced with a virgin carrier. Biofilm from a single carrier was carefully and completely scraped with a sterile pipet tip and resuspended in 2 mL RNA protect reagent (Qiagen, CA), then it was visually checked to ensure no residual biofilm on the carrier. Motor (Fisher, MA) with sterile tip (USA Scientific, FL) was used for the disaggregation of detached biofilm biomass.

DNA was extracted using Qiacube with the DNeasy mini kit (Qiagen, CA) and extracted DNA was tested by Nanodrop Lite spectrophotometer (Thermo Scientific, WI) to ensure good DNA quality for the following molecular experiments.

4.2.4 Stable isotope probing

For each sample, 5 µg extracted DNA were added to a mixture of 7.163 M cesium chloride (CsCl) and gradient buffer (0.1 M Tris, 0.1M KCl and 1 mM EDTA) set to a final density of 1.725 g/mL before centrifugation for 60 h at 20 °C and 40,000 rpm with vacuum, using a Vti 65.2 rotor and a L8-80M ultracentrifuge (Beckman Coulter). Fractionation of CsCl gradients was done using a syringe pump flow rate set at 500 µL/min. Nine CsCl fractions, each of 500 µl, were obtained. 150 µl of DNA from

each fraction was precipitated by adding 450 µl of DNA/RNA free water and 120 µl of 7.5 M ammonium acetate, 2 µl of glycogen and 1,200 µl of 200 proof ethanol and left at room temperature overnight. DNA sample was added with 750 µl of 200 proof ethanol at the second day and left under 4 °C for 60 minutes before centrifugation at 13,000 g for 45 minutes at 20 °C. DNA pellets were washed with 1,000 µl of 70% (v/v) ethanol, centrifuged for another 20 minutes at 13,000 g, after which 100 µl of DNA/RNA free water was added to each sample. Total bacteria concentrations DNA recovered from previous step was quantified by quantitative polymerase chain reaction (qPCR) assays, using a CFX384 realtime PCR thermal cycler (BioRad Laboratories, Hercules, CA) with eubacterial 16S rRNA primers 1055F(5'-ATGGCTGTCGTCAGCT-3') and 1395R (5'-ACGGGCGGTGTGTAC -3') [165]. Standard curves for qPCR assays were generated via serial decimal dilutions of plasmid DNA containing specific target gene inserts. Melting curve for each qPCR assay was analyzed to confirm primer specificity. A density of 1.733 g/mL was applied as the boundary to separate each sample into the light and heavy parts for further molecular analysis.

4.2.5 16S rRNA gene amplicon sequencing

Using the Ion Torrent PGM platform chemistry and protocols, barcoded fusion primers with sequencing adaptors and a 1044F/1392R universal primer set were applied in each sample for multiplex sequencing. Prior to template preparation, quantification and qualification of amplified library were performed by bio-analyzer. Template preparation with DNA library followed by ion sphere particles (ISP)

enrichment was performed using the Ion OneTouch2 system following the manufacturer's instruction (Ion OT2 400 kit). The enriched ISP was loaded onto an Ion Torrent 3128 chip and run on an Ion Torrent PGM according to manufacturer's instructions (Ion PGM Sequencing 400 Kit). Ion Torrent Suite software was used for base calling, signal processing, and quality filtering of raw sequences. Mothur software was applied for post run bioinformatics analysis of the amplicon sequences.

4.3 Results and discussion

4.3.1 Impact of organic carbon on mainstream anammox process performance

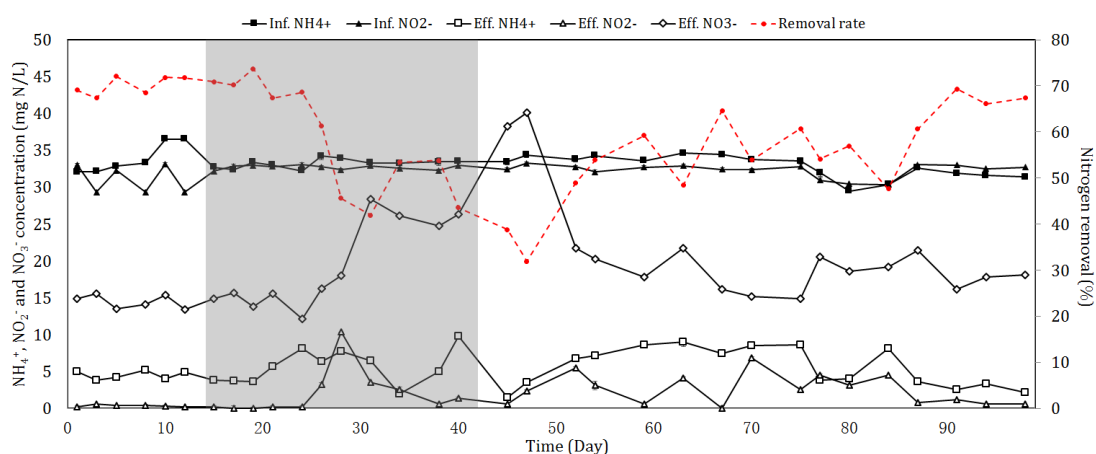


Figure 17 Performance of mainstream anammox MBBR during additional organic carbon period; results represent average and standard deviation of duplicate measurements.

The mainstream MBBR was operated without additional organic matter in 14 days, it demonstrated an average nitrogen removal of $70 \pm 2.0\%$. After 14 days, organic matter (Table 2) was introduced, the mainstream MBBR had a good performance initially. During the first week after introducing organic matter, nearly 100% removal

of nitrite was achieved and it showed an average total nitrogen removal of $71 \pm 2.6\%$ (Figure 17). However, negative impacts on process performance appeared after 20 days, dramatic loss of nitrogen removal was monitored with an average total nitrogen removal of $48 \pm 6.3\%$ in last two weeks of the organic matter dosing period. Ammonium contained in effluent gradually increased to 9.81 ± 0.0010 mg-N/L at day 24. The concentration of ammonium in effluent declined afterward to 1.94 ± 0.06 mg-N/L at day 34, but it increased again until the end of organic carbon dosing period. Residual nitrite in effluent went up dramatically after 24 days and decreased after 28 days, highest nitrite concentration of 10 ± 0.20 mg-N/L was monitored at day 28.

Studies have been reported that organic carbon could lead to adverse effects on AMX [72-74], hence the substrates (ammonium and nitrite) of anammox process could be accumulated due the impaired activity of AMX. A similar trend of ammonium concentrations in the effluent was also observed after organic carbon was introduced [85], that is a temporarily increase followed by a reduction. The contribution of ammonium assimilation by OHO could explain the reduced level of ammonium concentration in the effluent [166, 167]. On the other hand, the credit of denitrification is proposed to explain the reduction of nitrite concentration in the effluent after the short shock. With introduced organic carbon, OHO could be easily boosted utilizing nitrite as the electron donor and organic carbon as the electron acceptor to produce nitrogen gas. Moreover, nitrate production was enhanced with the presence of organic carbon, concentration of which was nearly doubled after 31 days. Although there was no aeration provided in the reactor, oxygen in the overhead space

could contribute to oxidation of nitrite to nitrate. It explained decrease of nitrite concentration along with increase of nitrate concentration in the effluent after 28 days. The Organic matter dosing was stopped after 42 days, process performance gradually recovered. Full recovery of the mainstream MBBR was demonstrated after 91 days, an average total nitrogen removal of $68 \pm 1.6\%$ was achieved.

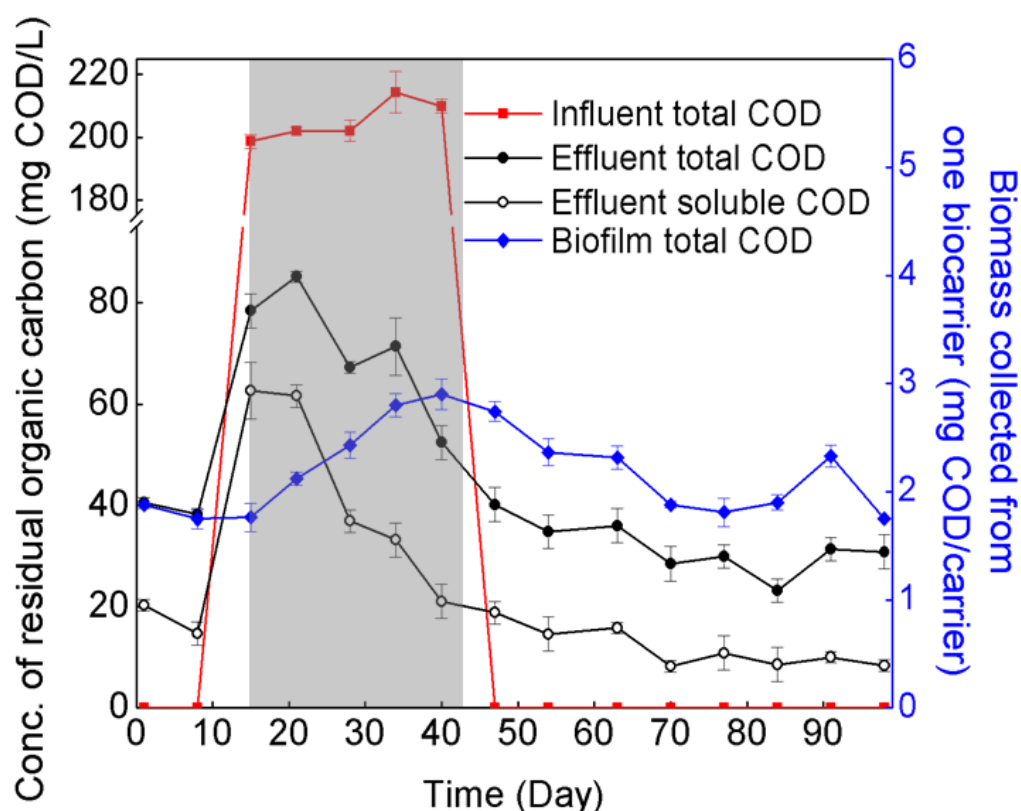


Figure 18 COD measurements of influent, effluent and collected biofilm from biocarrier, area shadowed with grey is organic matter dosing period; results represent average and standard deviation of duplicate measurements.

In pre-organic carbon dosing period, soluble inorganic carbon was dosed as the only carbon source, but both total COD and soluble COD in the effluent were demonstrated. The decay of cells and soluble microbial products contributed to the measured soluble COD in effluent. After organic matter was dosed, total COD

removal was around 60% initially and then enhanced during organic matter dosing period, achieving maximum total COD removal of 75% at day 40 (Figure 18). It was shown organic matter could be utilized as the electron donor by anammox bacteria to reduce nitrate and/or nitrite to ammonium [75]. Moreover, anammox bacteria can be enriched in the presence of acetate, and the enriched biomass can oxidize other organic matters [80]. Thus the metabolic diversity of AMX with the presence of organic carbon could result in an increasing COD removal. However, the organic carbon could compete with the ammonium as the electron donor for the electron acceptor nitrite, resulting a decreased nitrogen removal performance [75]. At organic-carbon-free condition, the anaerobic ammonium oxidation pathway could be re-expressed [58], which could explain the overall reversible impact of additional organic carbon on the process performance (Figure 17). Furthermore, heterotrophic bacteria have relatively shorter doubling time while doubling time of anammox bacteria are usually up to days [97, 98, 168], with sufficient supply of organic carbon source heterotrophs could be easily boosted and brought up the total COD removal.

On the other hand, COD measurements of samples collected from biocarriers gradually increased since organic matter dosing was initialized, which could be contributed by the overgrowing of heterotrophic bacteria and the adsorption of organic matter on the biofilm. Synthetic organic matter applied in this research contained both soluble and non-soluble COD, which could probably be attached on biocarriers. After organic carbon dosing was stopped after 42 days, soluble COD and total COD in effluent declined immediately without additional carbon supplying and

stable concentrations were monitored after 70 days (Figure 18). Similar trend was observed on COD measurements of biofilm from biocarriers, it slowly reduced after organic carbon dosing stopped and kept stable at a pre-organic carbon dosing level after 70 days (Figure 18).

4.3.2 Anammox and denitrification activity tests

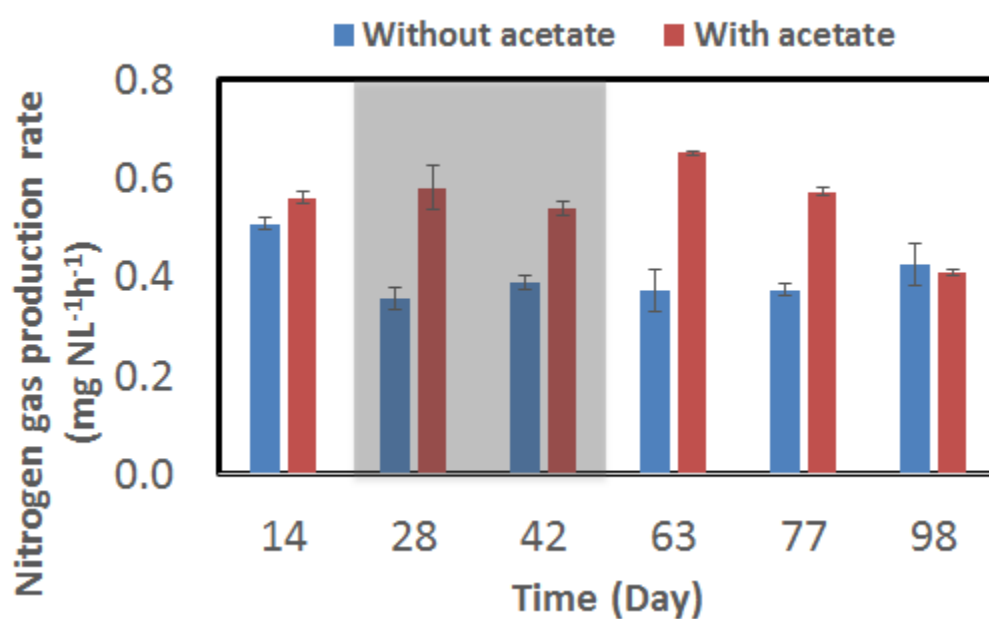


Figure 19 Nitrogen gas production rates of anammox activity tests without acetate and with acetate, area shadowed with grey is organic matter dosing period; results represent average and standard deviation of duplicate experiments.

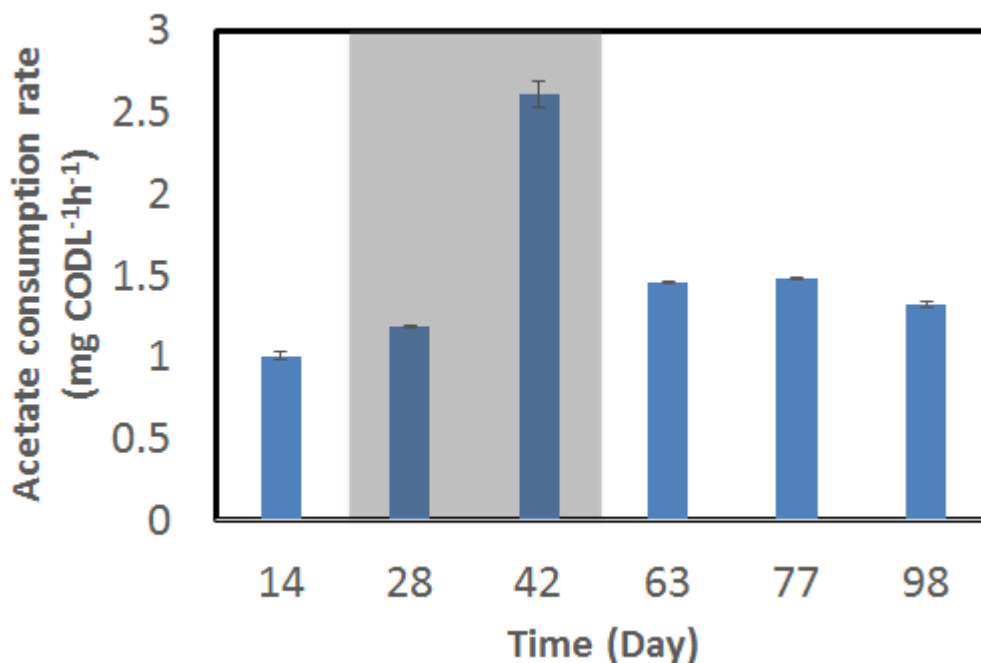


Figure 20 Acetate consumption rates of denitrification activity tests, area shadowed with grey is organic matter dosing period; results represent average and standard deviation of duplicate experiments.

When anammox activity tests without acetate were conducted before, during and after organic carbon dosing, a noticeable negative impact was observed. The nitrogen gas production rate decreased from 0.51 ± 0.010 mg-N/L h at day 14 to 0.36 ± 0.020 mg-N/L h at day 28 (Figure 19). However, after discontinuing organic carbon dosing on the mainstream MBBR, nitrogen gas production rate was gradually enhanced. In batch test B 错误!未找到引用源。 , acetate was applied along with ammonium and nitrite to explore anammox activity during and after organic carbon dosing. During the first three sets of batch tests B (day 14, day 28 and day 42), nitrogen gas production rate was demonstrated at 0.56 ± 0.010 mg-N/L h, 0.58 ± 0.050 mg-N/L h and 0.54 ± 0.010 mg-N/L h respectively (Figure 19). However, an average nitrogen removal of $50 \pm 7.5\%$ was still achieved at the end of organic carbon dosing period, revealing that nitrogen gas produced by denitrification probably contributed to the

total nitrogen gas production. Moreover, the gap of nitrogen gas production rate between batch test A and B did not decrease until day 77 (Figure 19), indicate the considerable potential of denitrification activity even after organic carbon dosing was stopped.

In batch test C, to exclude the influence of residual nitrite, acetate consumption rate was employed to evaluate the activity of denitrification. Denitrification activity was greatly enhanced in batch tests conducted during organic carbon dosing period (Figure 20), which is to be expected since sufficient organic carbon source could promote the growth of heterotrophic bacteria. Moreover, high activity of denitrification was still demonstrated even after 42 days, reduction of denitrification activity was only observed at day 98. This trend was verified by what observed in batch test B, the potential of denitrification was still substantial after organic carbon dosing was stopped.

4.3.3 Shift of microbial ecology before, during and after organic carbon dosing period

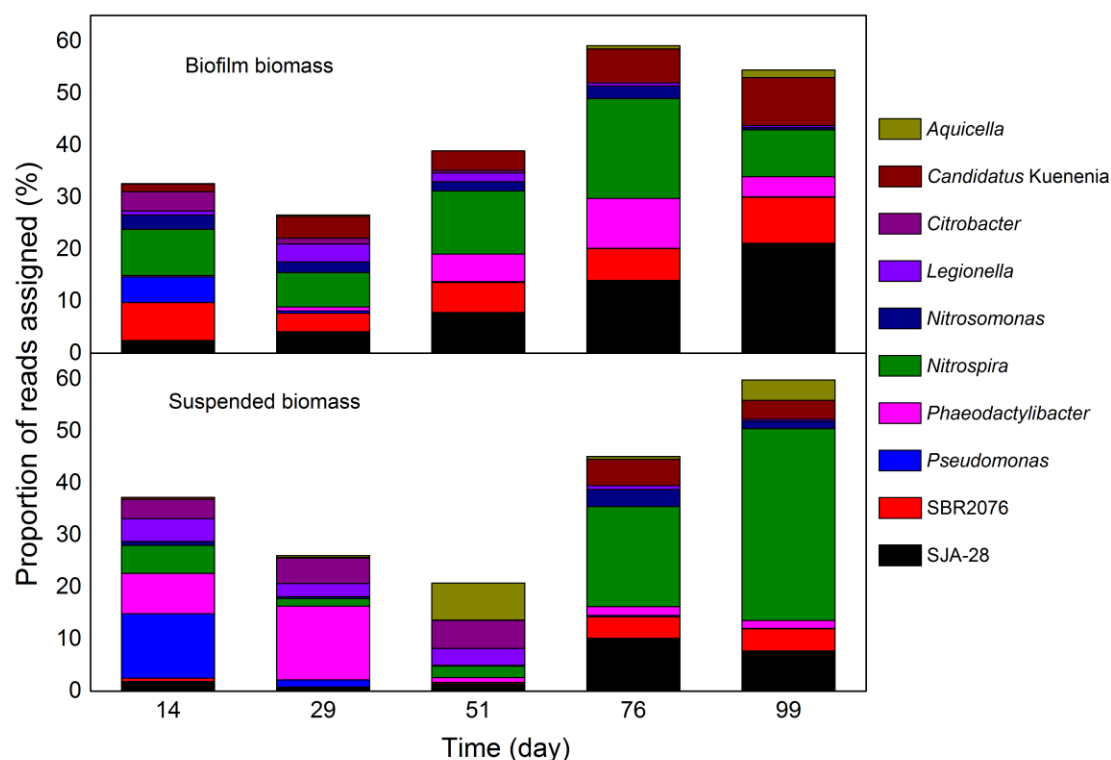


Figure 21 Taxonomic classification of the bacterial communities in both biofilm and suspended biomass of the mainstream MBBR at genus level (based on 16S rRNA gene sequencing, top 10 genera shown), samples before (day 14), during (day 29) and after (day 51, 76 and 99) organic matter dosing period were conducted.

Ion Torrent PGM platform chemistry and protocol were performed to identify the overall bacterial community composition from biofilm and suspension.

The results showed that 1.43% of reads were assigned to *Candidatus* “Kuenenia” in the biofilm sample from day 14, the proportion of reads increased to 4.18% at day 29, which was dosed with organic matter (Figure 21). After dosing was ended, the proportion of reads assigned to *Candidatus* “Kuenenia” still gradually built up and achieved 9.2% at day 99 (Figure 21). Moreover, fractions of OHO, such as *Citrobacter*, *Leginoella*, dramatically decreased after organic carbon dosing was stopped (Figure 21). It was discussed above that the COD of biofilm was gradually

elevated during organic carbon dosing period (Figure 18), implying the physical adsorption of organic matter and the accumulated bacterial community. Introduction of organic carbon could increase EPS contents, which contributed to a stable granulation structure and benefited accumulation of AMX [169, 170]. Thus AMX was selectively enriched along with the growing biofilm in the biocarrier after organic carbon was introduced. The enhancement of AMX dominance was further observed after the termination of organic carbon dosing, which could be explained by the wash-out of OHO and selective retention of AMX. *Nitrospira* spp. related NOB was identified in both biofilm and suspension samples. Percentage of reads assigned to *Nitrospira* spp. in biofilm samples declined slightly at day 29 and increased up to 20% at day 76, after which a significant loss was demonstrated at day 99 (Figure 21). Along with reduced fraction of *Nitrospira* spp. in the biofilm at day 99, a considerable increase of *Nitrospira* spp. proportion was confirmed in suspension at the same day, which could be explained by the detachment of *Nitrospira* spp. related NOB from biofilm into suspension. Selective retention of AMX in the biofilm competed with *Nitrospira* spp. related NOB for nitrite, resulting in the out-selection of *Nitrospira*.

Low fractions of *Nitrosomonas* were identified in all biofilm and suspension samples, but there was no significant change verified during whole time series experiments. Aerobic pathway of *Nitrosomonas* could hardly be performed with no aeration provided in the mainstream MBBR, but anaerobic pathway of *Nitrosomonas* was reported using pyruvate as an electron donor and nitrite as an electron acceptor [171]. Thus *Nitrosomonas* could be still active through the anaerobic metabolism, or just

stay entirely inactive in the system. Moreover, the mainstream MBBR reactor employed in this research was enriched from a nitrification/anammox sidestream MBBR reactor, the presence of *Nitrosomonas* was initially seeded by the inoculum from sidestream MBBR reactor. Furthermore, uncultured bacteria SBR-2076 and SJA-28 had a considerable increase during whole time series experiments, these two genera belong to Phylum *Chloroflexi* and *Chlorobi* respectively, which are commonly reported co-existence with anammox bacteria [172-174].

4.3.4 DNA gradient ultracentrifugation and identification of labeled 16S rRNA genes

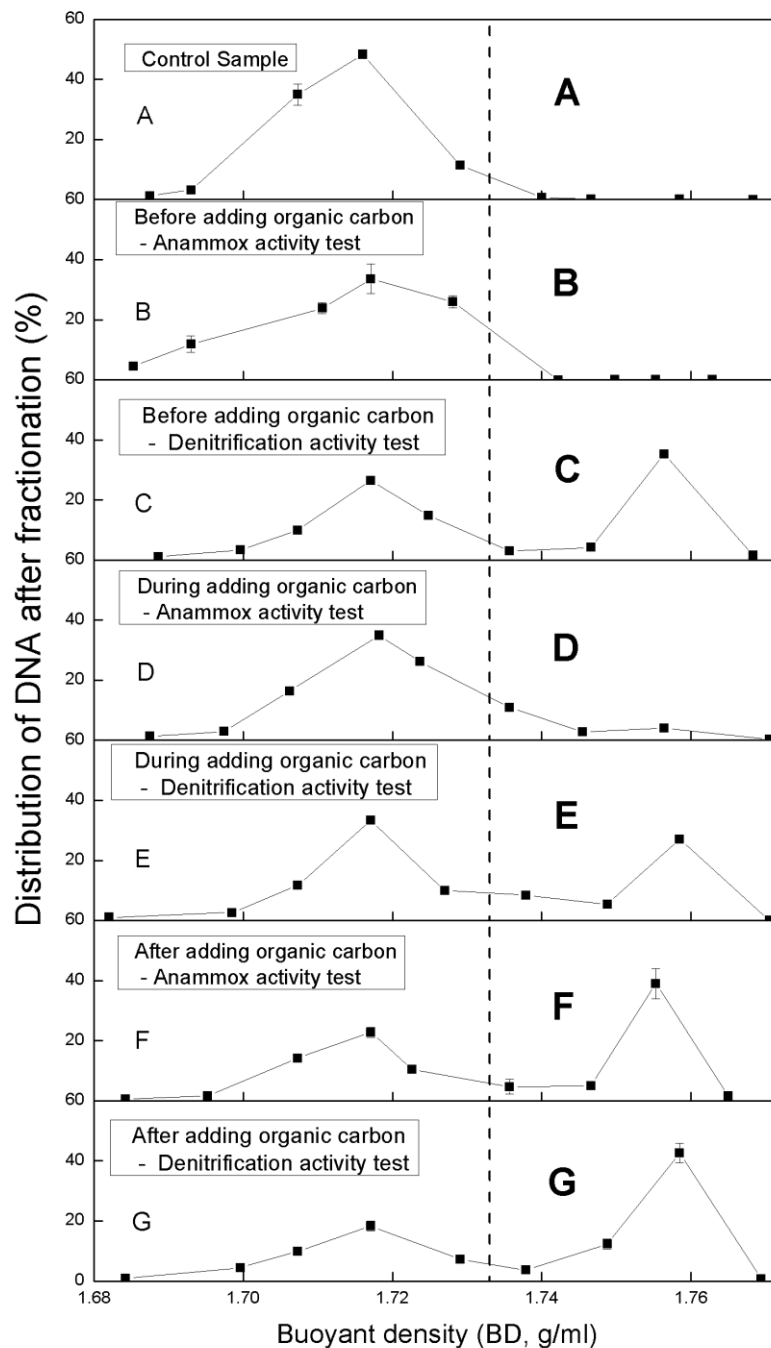


Figure 22 Percentage of each SIP fraction based on qPCR analysis of Eubacteria 16S rRNA gene. Results represent average and standard deviation of triplicate measures. (A) unlabeled genomic DNA from control sample; (B, D and F) ^{13}C - genomic DNA from anammox activity batch tests (batch test B) samples at Day 14, 42 and 77. (C, E and G) ^{13}C - genomic DNA from denitrification activity batch tests (batch test C) samples at Day 14, 42 and 77.

After fractionation, qPCR analysis targeting the Eubacteria 16S rRNA gene was conducted to evaluate the proportion of DNA for each fraction (Figure 22). It was easily observed and to be expected that nearly all DNA from the control sample was located in the light part, there was barely any DNA remaining in heavy part. DNA samples from the denitrification activity tests all showed clear peaks at heavy fractions, which suggested the assimilation of ^{13}C by microorganisms. On the other hand, DNA samples from anammox activity tests at day 77 also showed a considerable amount of DNA located in the heavy fractions, but only a small amount and almost no DNA samples were identified from batch tests operated at day 14 and 42 respectively.

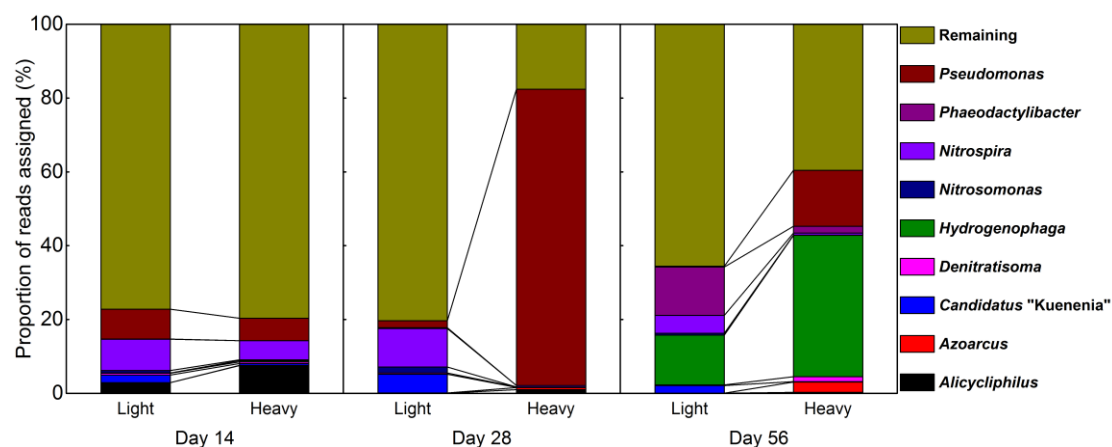


Figure 23 Taxonomic classification of the bacterial communities at genus level for samples from batch test B after SIP (based on 16S rRNA sequencing, abundant genera involved in N cycle were shown)

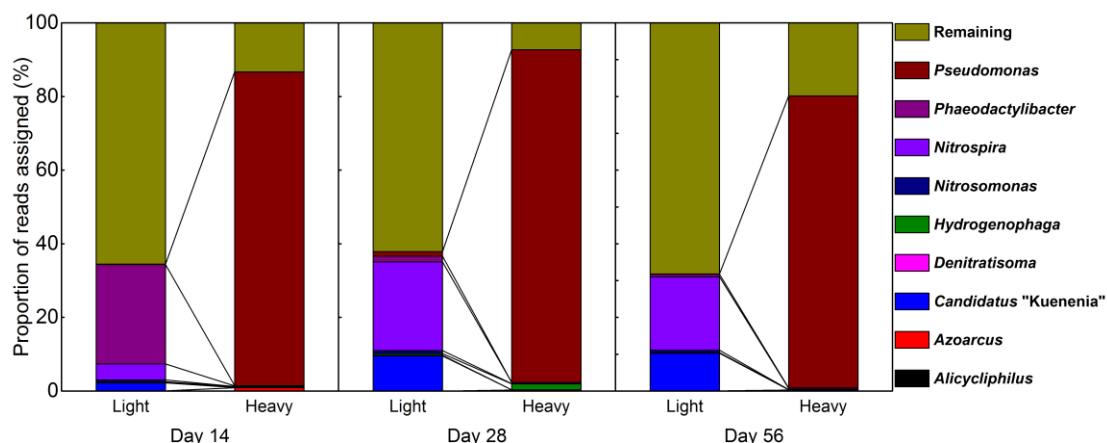


Figure 24 Taxonomic classification of the bacterial communities at genus level for samples from batch test C after SIP (based on 16S rRNA sequencing, abundant genera involved in N cycle were shown)

Proportions of reads assigned to *Candidatus* “Kuenenia” in heavy part DNA of all samples were notably lower than that in light part DNA (Figure 23 and Figure 24), suggesting the disability of ^{13}C -labeled acetate assimilation by *Candidatus* “Kuenenia” in this case. The most abundant functional group revealed after batch test B and C was denitrifier, including *Alicyclophilus*, *Azoarcus*, *Denitratisoma*, *Hydrogenophaga*, *Phaeodactylibacter* and *Pseudomonas* in genus level. Among these denitrifiers, *Pseudomonas* was the most dominant genus in the heavy parts of samples from batch test C, and the monitored significant difference of fractions between the light and heavy parts was contributed by assimilating ^{13}C -labeled acetate. The capability to assimilate acetate by *Pseudomonas* was also confirmed by other researchers [175, 176], it has been further reported that assimilation of acetate could be achieved by *Hydrogenophaga* as well [177]. Results in this study showed that fractions of *Hydrogenophaga* in samples from batch test B at Day 56 had a relatively greater value in the heavy part (Figure 23), suggesting the ability of acetate assimilation.

Moreover, other than denitrification, a second pathway was suggested for *Hydrogenophaga* under aerobic condition, using the oxidation of hydrogen gas as an energy source and inorganic carbon as the carbon source [178]. Indeed, hydrogen gas could be supplied by fermentation process under anaerobic condition, but the non-negotiable need for oxygen as the terminal electron acceptor eliminate the possibility of this pathway. Lastly, *Candidatus* “Kuenenia was detected in all heavy part samples, but the proportions of reads assigned were not significant, suggesting the ability of acetate assimilation cannot confirmed in this study.

4.4 Conclusions

In summary, the anammox process was suppressed after dosing organic carbon into the mainstream MBBR, demonstrated by both decreased average ammonium removal in the reactor and lower ammonium consumption rates in the batch tests. Meanwhile, the dosing of organic carbon promoted heterotrophic denitrification activity, which compensated for nitrogen gas production. In addition, this newly enriched mainstream anammox MBBR still achieved over 70% of ammonium removal and 65% of nitrite removal during organic carbon dosing, and the reactor fully recovered after termination of organic carbon dosing. SIP and 16S rRNA gene sequencing were applied to investigate the anabolism of functional groups. Results showed the incapability of anammox to assimilate acetate, the community assimilating ¹³C-labeled acetate was mainly assigned to denitrifiers. Presence of denitrifiers was observed in mainstream MBBR and stayed inactive without sufficient organic carbon, but it was boosted after additional organic carbon dosing. These results demonstrate

that the mainstream anammox process as tested in this study was resilient to varying influent loads of organic carbon.

Chapter 5. Impact of Wet Weather Flow on a Mainstream Anammox Moving Bed Biofilm Reactor (MBBR)

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5.1 Introduction

Since the first time AMX were detected 20 years ago in Delft University [110], it has been widely applied in WWTPs. By fully applying anammox process in WWTPs, 60% of the oxygen demand reduction could be achieved [111], that is approximately 50% of electricity consumption in WWTP[91]. However, most of the anammox applications are employed in the sidestream, which only contains 15%-20% of total nitrogen loadings of a WWTP. The bulk of it still needs to be removed from the mainstream [118]. Research interests thus have shifted from sidestream to mainstream applications.

In mainstream, the fluctuant flow rate in the mainstream, which is highly dependent by weather, is considered an essential factor having negative impacts on anammox biofilm system. During wet weather, WWTPs will face a peak flow rate excessively higher than usual. According to wet weather operating practices of WWTPs in New York City, plants are designed to provide primary treatment to wet weather peak flow of twice design average dry weather flow, and secondary treatment to one and a half times average dry weather flow. The resulting high peak in wet weather may lead to the detachment of biomass from biocarriers, low concentrations of functional microorganism in suspension and unexpected variability of sewage composition. Once the excessive detachment appeared, limited by the particularly low maximum growth rate of anammox [38], it will be extremely time consuming to have anammox bacteria recovered. Meanwhile, instead of anammox bacteria, the microorganisms with high growth rate, like AOB and NOB [179, 180], will occupy the biocarrier more

efficiently. The increasing fraction of NOB in biofilm will likely result in a long-term deterioration of process performance [71].

5.2 Material and methods

5.2.1 Lab-scale reactors configuration and operation



Figure 25 Setup of mainstream MBBR and sidestream MBBR during enrichment phase

A newly enriched 6 L lab-scale mainstream anammox MBBR was coupled with a 6 L lab-scale sidestream anammox MBBR (Chapter 2). Both these two MBBRs contained 2000 kaldnes K1 biocarriers, achieving a total volumetric fill capacity of 33%.

Reactor performance was monitored by measuring the concentrations of ammonia (potentiometry, Thermofisher, PA), nitrite (diazotization-colorimetry, RICCA, IN), nitrate (potentiometry, Fisher, MA) and hydroxylamine (colorimetry) [95] each sampling day. Reactor dissolved oxygen (DO) concentrations were measured by Clark type electrodes (05643-00, Cole Parmer, IL), interfaced to a DO monitor (01972-00, Cole Parmer, IL). Biomass concentrations in the biofilm and suspension were measured in the biofilm phase and suspension phase with Hach high-range COD digestion vials (20-1500 mg/L, Hach Inc., CA).

5.2.2 Lab-scale reactors operation in wet weather period

Simulated wet weather flow was added into the mainstream reactor for three weeks (day 327-333, day 341-347 and day 397-403) during the entire study. Additional wet weather flow chart (Table 4) and recipe of the synthetic wet weather (Table 5) are shown as follows.

Table 4 Flow rate chart in wet weather phase

Time period	Flow rate (liter/day)	Time period	Flow rate (liter/day)
Day 44-50	4.00	Day 58-64	7.73
Day 114-120	7.73		

Table 5 Ion distribution in synthetic wet weather flow

Cation	Conc. ($\mu\text{mol/L}$)	Cation	Conc. ($\mu\text{mol/L}$)	Anion	Conc. ($\mu\text{mol/L}$)
Na^+	5.63E+01	Mo^{7+}	3.34E-03	NO_3^-	2.23E+01
Ca^+	5.24E+00	Cu^{2+}	4.44E-02	Cl^-	3.02E+01
K^+	1.53E+00	Zn^{2+}	1.01E-01	PO_4^-	1.00E-01
NH_4^+	3.50E+01	Ni^{2+}	9.37E-03	SO_4^{2-}	11.2E+01
Mg^{2+}	2.06E+00	Fe^{2+}	1.50E-01	CO_3^{2-}	3.00E+01
Mn^{2+}	7.66E-02	Co^{2+}	3.39E-04		

5.2.3 Lab-scale sample collection and DNA extraction

For the lab-scale reactor, biomass samples from suspension and biofilm were collected each sampling day and stored at -80°C for subsequent molecular processing. For suspension biomass sampling, 30 mL suspension from each reactor was taken and transferred into a 50 mL sterile tubes (Corning, MA) with 50 mL sterile pipet. After centrifuge (Beckman Coulter, NJ) at 4 °C and relative centrifugal force of 8000G for 10 minutes, the supernatant was discarded and biomass was resuspended in 1 mL RNA protect reagent (Qiagen, CA). For biofilm biomass sampling, one carrier was sacrificed in each reactor and replaced with a virgin carrier. Biofilm from a single carrier was carefully and completely scraped with a sterile pipet tip and resuspended in 2 mL RNA protect reagent (Qiagen, CA), then it was visually checked to ensure no biofilm residual on the carrier. Motor (Fisher, MA) with sterile tip (USA Scientific, FL) is applied for the disaggregation of detached biofilm biomass.

DNA was extracted using Qiacube with the DNeasy mini kit (Qiagen, CA) and extracted DNA was tested by Nanodrop Lite spectrophotometer (Thermo Scientific, WI) to ensure good DNA quality for the following molecular experiments.

5.2.4 Amplicon sequencing

Using the Illumina sequencing platform chemistry and protocols, 16S rRNA regions were targeted and amplified for analysis from both DNA and RNA extracted from each reactor over the course of enrichment (Illumina Inc, San Diego, CA). Data was processed using the mothur software program (version 1.36.1)[96]. Contigs were formed using the paired Illumina reads and filtered based on size (275-325 bp), homopolymeric regions (maximum 8 bp), and ambiguous regions (maximum 0 bp).

Mothur software was applied for post run bioinformatics analysis of the amplicon sequences.

5.3 Results and discussion

As discussed in Chapter 2, the sidestream MBBR was coupled with the mainstream MBBR to provide AMX as the inoculum as well as ammonium, nitrite and other trace elements as the substrates. In the post-enrichment phase, two MBBRs were kept coupled when synthetic wet weather flow was introduced. During this whole wet weather experiments periods, the monitored performance of the sidestream MBBR was slightly fluctuated, especially for the nitrite in effluent, achieving an average total nitrogen removal of $83 \pm 5.6\%$ (Figure 26). There were days that residual nitrite in the effluent exceeded 50 mg-N/L, which could suppress the activity of anammox. It was reported nitrite has a significant inhibition on the activity of anammox when the concentration is over 40 mg-N/L [181]. Prior research has demonstrated that nitrite concentration as low as 5 mg-N/L could already be inhibitive [40]. Moreover, it was recently reported $\text{NO}_2^-/\text{NH}_4^+$ ratio of 1.146 is an optimum value for the anammox process. High concentrations of nitrite in the sidestream MBBR resulted in an unbalanced ratio of $\text{NO}_2^-/\text{NH}_4^+$, which would excessively provide the electron acceptor while being insufficient for the electron donor.

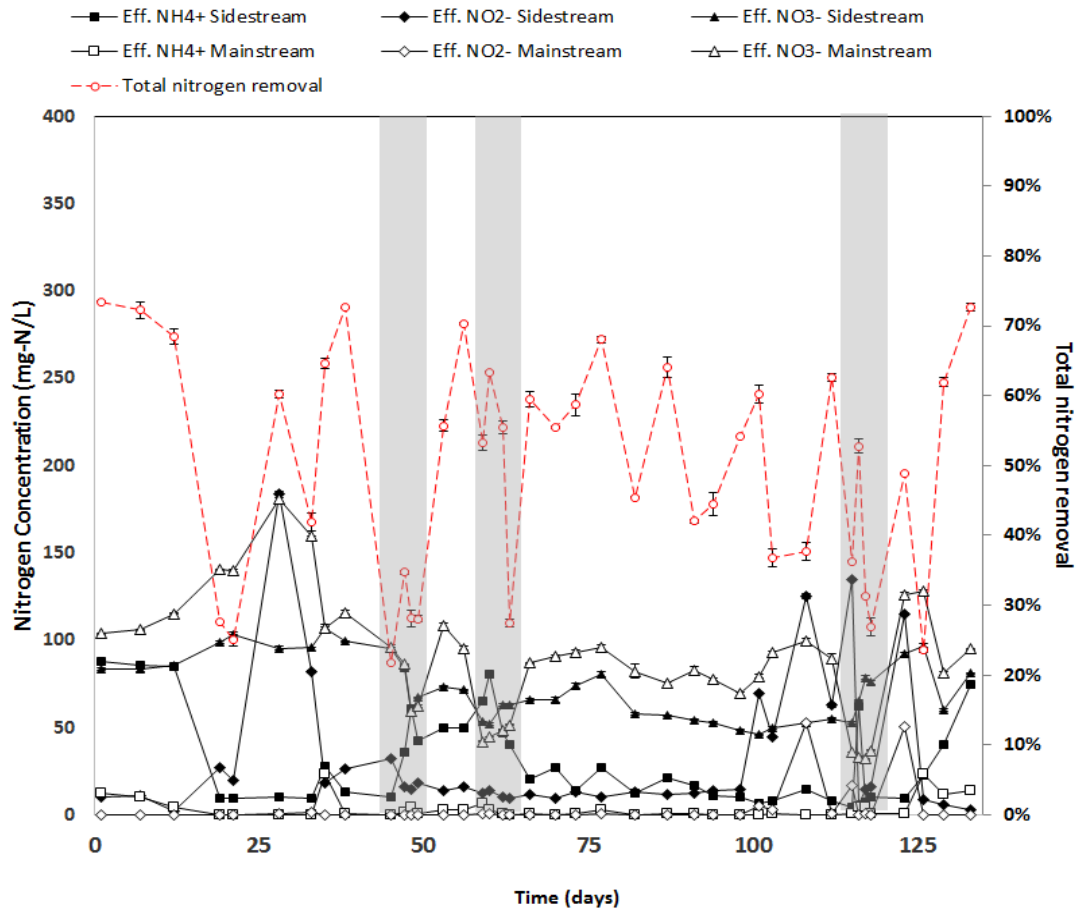


Figure 26 Performance of the sidestream MBBR, additional synthetic wet weather flow was provided along with effluent of the sidestream to feed the mainstream (area shadowed with grey); results represent average and standard deviation of duplicate measurements.

Performance of the mainstream MBBR was monitored during the entire wet weather experiment. Despite the days with massive residual nitrite in the influent, total nitrogen removal was kept at a relatively high level with no wet weather flow introduced (average total nitrogen removal of $53 \pm 14\%$). However, reactor performance was significantly impaired in three wet weather events (average total nitrogen removal of $38 \pm 13\%$). Three hypotheses are proposed to explain the impaired performance of the mainstream MBBR during the wet weather event. When wet weather flow was introduced, the concentrations of ammonium and nitrite in the

mainstream MBBR were diluted along with a reduced HRT; this lead to a lower nitrogen consumption rate of anammox bacteria. Moreover, the suspended biomass from the mainstream MBBR was highly dependent on the inoculum from the sidestream, and was in parallel diluted when the wet weather condition was imposed. Thus reduced biomass in the suspension could draw back the efficiency of nitrogen removal. Ultimately, detachment of biomass from the biocarrier could be magnified when HRT is lower. This was especially the case for the second and third wet weather events, HRT dramatically declined from 1.5 days to 0.51 days. However, the impact of wet weather event was reversible, which was demonstrated by the quick recovery of reactor performance after every wet weather event.

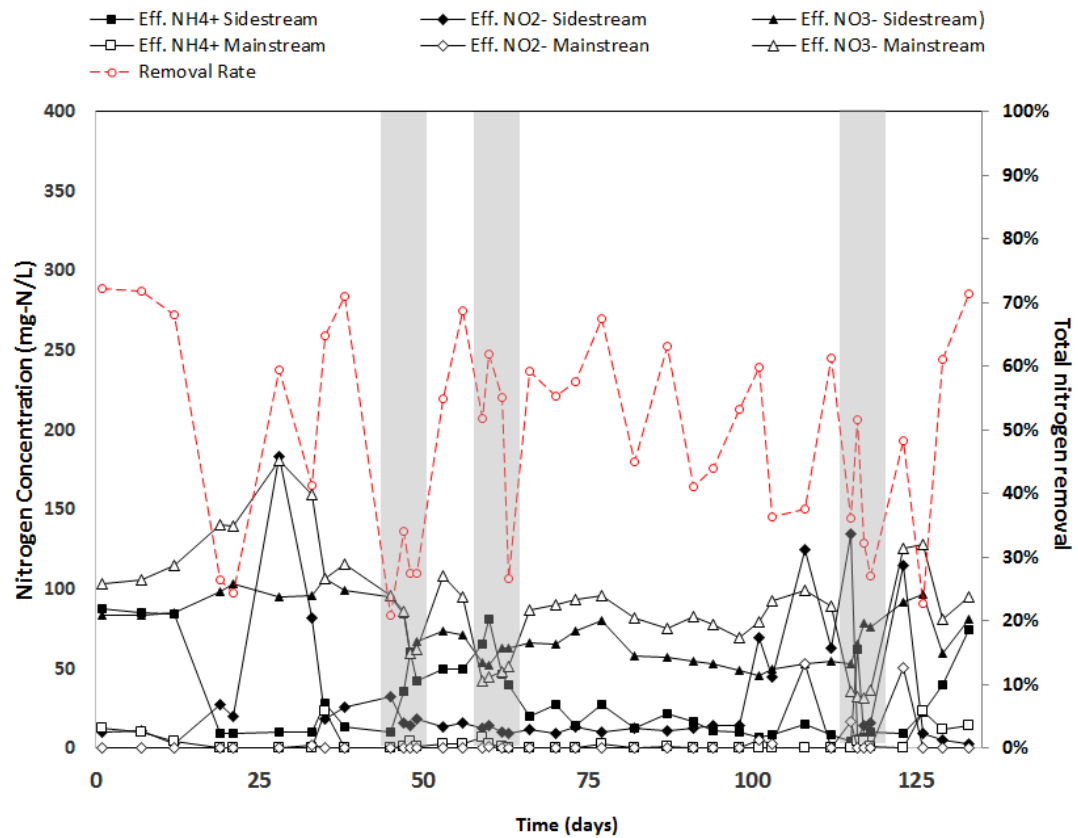


Figure 27 Performance of the mainstream MBBR, additional synthetic wet weather flow was provided along with effluent of the sidestream to feed the mainstream (area shadowed with grey); results represent average and standard deviation of duplicate measurements.

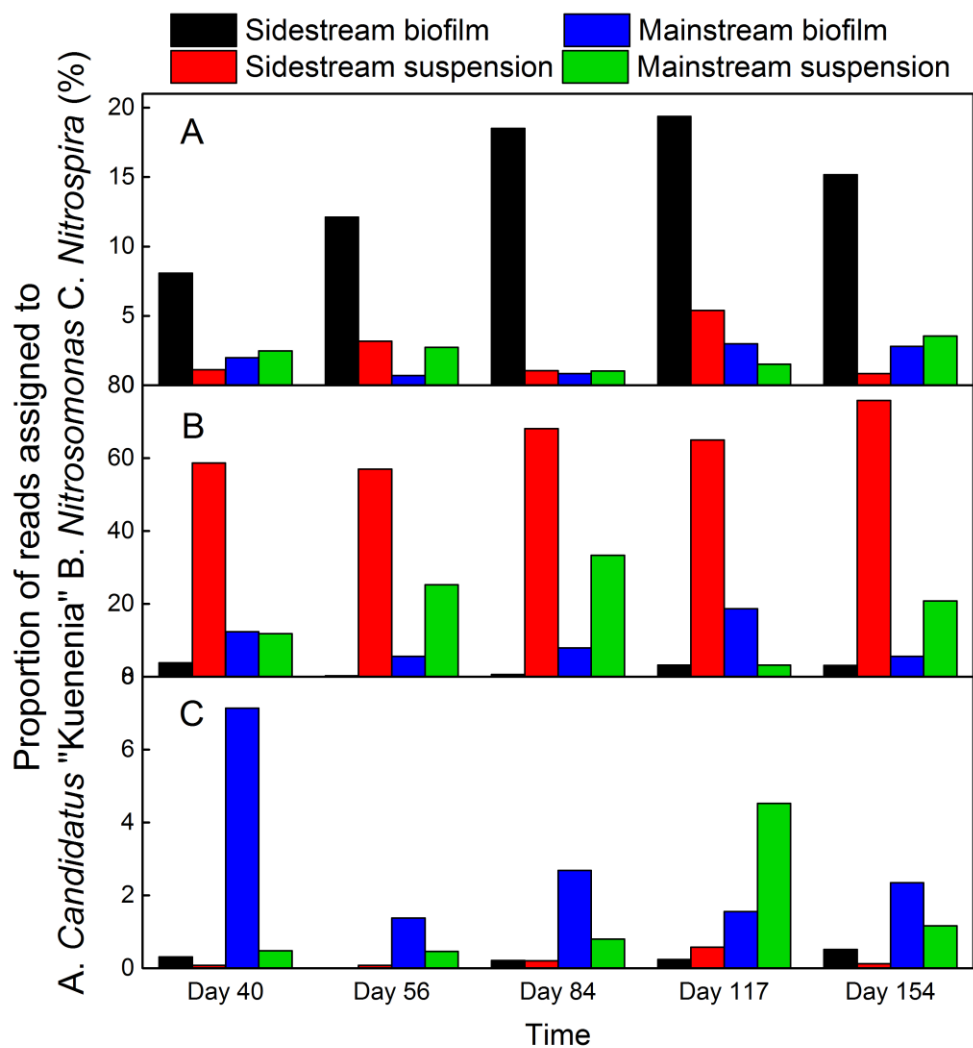


Figure 28 Proportion of reads assigned to A. *Candidatus* "Kuenenia" B. *Nitrosomonas* C. *Nitrospira* in both biofilm and suspended biomass of the coupled mainstream-sidestream system (based on 16S rRNA gene sequencing)

Illumina platform chemistry was applied to identify the overall bacterial community composition of the mainstream and sidestream reactors over the wet weather period using 16S rRNA gene sequencing. In mainstream suspension, up to 33% of reads was assigned to *Nitrosomonas* in mainstream suspension, but it dramatically dropped

down to 3.2% at Day 117 due to the additional wet weather flow (Figure 28). During wet weather period, HRT of the mainstream anammox MBBR was decreased to 0.51 days, while HRT was regularly maintained at 1.5 days. Even though the constant flow from sidestream suspension was providing *Nitrosomonas* to the mainstream anammox MBBR, fraction of which would still be hardly maintained without the support of aeration. However, proportions of reads assigned to AMX remained unaffected during wet weather period in the biofilm, showing the strong attachment of AMX to the biocarrier. In the geometry structure of the biocarrier, out-layer is mainly occupied by AOB [99, 100], while inner-layer contains most of AMX [100]. Thus shortly changed HRT might only have limited influence on the inner-layer microbial structure. *Nitrospira* was detected in the suspended biomass of mainstream MBBR as well as in the biofilm biomass. An up trend of fraction of *Nitrospira* was monitored at Day 117 and 154, which was in parallel with the fraction of reads assigned to *Nitrospira* in the suspended biomass from the sidestream reactor. The special operational structure of coupling for two mainstream MBBRs led to the analogous molecular microbial ecology in the suspended biomass in the whole system. However, accumulation of *Nitrospira* was not confirmed in the biofilm biomass in the mainstream MBBR, suggesting the restricted influence during the wet weather flow. With significant reduced HRT, there was not evidence inspected for the loss of *Candidatus* “*Kuenenia stuttgartiensis*”, indeed, fraction of which was enhanced after Day 117. It could be explained by the loss of out-layer biomass, which provided extra space for the accumulation of AMX.

5.4 Conclusions

In sum, the reliability of a newly enriched mainstream anammox MBBR during wet weather condition was examined and tested successfully in this study. Impairment of reactor performance was monitored when a synthetic wet weather flow was imposed; however, a quick recovery was exhibited after each wet weather period, suggesting the reversible impact of reduced HRT and the trace elements from rainwater. A shift of molecular microbial ecology for the biomass in the suspension of mainstream MBBR was demonstrated in parallel with that of the sidestream MBBR due to the structure of coupling for these two MBBRs. However, a fraction of *Candidatus* “*Kuenenia stuttgartiensis*” related AMX was maintained unchanged in the first two wet weather events, and yet was enhanced after the third wet weather event, showing that the mainstream MBBR is a robust system even in wet weather conditions.

Chapter 6. Long-term Monitoring of Full-Scale Mainstream and Sidestream Deammonification Processes

6.1 Introduction

Nitrogen removal from wastewater treatment is of great value because of the significant adverse environmental impact of ammonia/ammonium on receiving water body, such as eutrophication. For decades, nitrogen removal in WWTPs is mostly performed by nitrification and denitrification, both of which are costly and energy consuming.

Conventionally, influent ammonia is oxidized to nitrite and then to nitrate by two distinct types of autotrophic nitrifying bacteria (ammonia oxidizing bacteria, AOB, and nitrite oxidizing bacteria, NOB). There are four among AOB (*Nitrosomonas*, *Nitrospira*, and *Nitrosovibrio*, and *Nitrosococcus*) [11], and four contain NOB (*Nitrobacter*, *Nitrococcus*, *Nitrospina*, *Nitrotoga* and *Nitrospira* [17-21]. Among these identified NOB, *Nitrotoga*, *Nitrospira* and *Nitrobacter* are most commonly studied and observed in municipal wastewater systems. [20-24]. A recent study showed *Nitrospira* spp. have not just higher affinities for nitrite and oxygen, but also a higher biomass yield and energy transfer efficiency relative to *Nitrobacter* spp. [25]. In a word, compared with conventionally studied *Nitrobacter* and *Nitrotoga* related NOB, *Nitrospira* related NOB are more abundant at low temperature in mainstream processes of wastewater treatment plants. Therefore, application of the anammox process in the mainstream needs to achieve out-selection of *Nitrospira* spp. rather than other NOB. Furthermore, nitrification was generally considered to be carried by two separate groups of microorganisms. However, complete oxidation of ammonia

into nitrate by a single microorganism was predicted in 2006 [86]. In 2015, the presence of such organisms was discovered within the *Nitrospira* genus [87, 88].

After nitrification process, denitrification is accomplished by utilizing organic carbon as the electron donor and extra organic carbon is commonly required to ensure a low nitrate residual in the effluent. Furthermore, this conventional process has an additional environmental impact, because of high biomass production [29] and greenhouse gas emission, especially N_2O [30, 31]. As a recently established technology, the anammox process is an environmental friendly, cost-effective biological removal process [56, 70]. In biological removal of nitrogen and organic matter, the aeration provided accounts for about 50% of electricity consumption in WWTPs [182]. Removal of nitrogen by anammox bacteria (AMX) is based on completely autotrophic oxidation of ammonium with nitrite as the electron acceptor, which offers advantages of reduced sludge production and less cost of consumption aeration and no organic carbon is needed [32-34].

Anammox processes have been implemented successfully in over 100 full-scale plants [7]. Most of these applications were implemented with high strength ammonia wastewater treatment in sidestream systems of municipal WWTPs [7]. However, as sidestream only accounts for less than 30% of total nitrogen loading in WWTPs, more attention has been attracted to the implementation of the anammox process in mainstream waters. However, reliable autotrophic nitrogen removal has not been well developed under typical mainstream sewage treatment conditions of high COD/N ratio and a relatively low temperature [69]. It was demonstrated that lowering of the

temperature in a nitrification-anammox MBBR resulted in decreasing anammox activity, but there was no major qualitative or quantitative changes in the microbial community [183]. Other research also reported that fading of anammox activity was observed in a MBBR reactor under lower temperature along with accumulated nitrite and stable microbial community, but loss of anammox population was confirmed in a SBR reactor after temperature was reduced [157]. Based on different applied treatment processes, the COD/N ratio in the influent of anammox process could vary between 1/1 and 10/1 [40, 81, 82].

The overarching objective of this study was to characterize the performance and microbial ecology of a full-scale process that transitioned from conventional towards mainstream-deammonification based BNR, during the course of over two years. Specific objectives included tracking the extent of nitrogen removal and the relative microbial concentrations as a function of operating conditions in the treatment process as well as the hydrocyclones, which were instituted to selectively enrich for AOB and anammox, while concurrently out selecting NOB.

6.2 Materials and methods

Field samples were taken and stored in a -80°C freezer after treating with RNA protect reagent (Qiagen, CA). Samples were shipped to our lab (Columbia University) on dry ice. DNA was extracted using Qiacube with the DNeasy mini kit (Qiagen, CA). The quantity and quality of the extract were tested using a Nanodrop Lite spectrophotometer (Thermo Scientific, WI) prior to subsequent molecular analyses. Quantitative polymerase chain reaction (qPCR) assays were performed to evaluate the

concentrations of total bacteria, AOB, *Nitrospira* spp. related NOB, *Nitrobacter* spp. related NOB, and AMX using an iQ5 realtime PCR thermal cycler (BioRad Laboratories, Hercules, CA) (Table 6), Standard curves for qPCR assays were generated via serial decimal dilutions of plasmid DNA containing specific target gene inserts. Melting curve for each qPCR assay was analyzed to confirm primer specificity.

Table 6 qPCR assays employed in this study

Target group	Primer/probe	Sequence (5'-3')	Ta(°C)	Reference
Eubacteria	1055F	ATGGCTGTCGTCAGCT	53	[165]
	1395R	ACGGGCGGTGTGTAC		
AOB	amoA-1F	GGGGTTTCTACTGGTG GT	57	[184]
	amoA-2R	CCCCTCKGSAAAGCCT TCTTC		
AMX	Pla46F	GGATTAGGCATGCAA GTC	55	[185]
	Anammox667R	ACCAGAAGTTCCACTC TC		
<i>Nitrospira</i> spp.	Nspra-675f	GCGGTGAAATGCGTA GAKATCG	58	[186]

	Nspr-746r	TCAGCGTCAGRWAYG		
		TTCCAGAG		
	Nspra-723Taq	CGCCGCCTTCGCCACC		
		G		
<i>Nitrobacter</i>	Nitro1198f	ACCCCTAGCAAATCTC	58	[186]
spp.		AAAAAACCG		
	Nitro1432r	CTTCACCCCAGTCGCT		
		GACC		
	Nitro1374Taq	AACCCGCAAGGGAGG		
		CAGCCGACC		

6.3 Results and discussion

The overall operation period was classified into five phases based on operating conditions imposed on the process.

6.3.1 Sidestream deammonification processes

Two sidestream deammonification processes were operated to provide seed biomass for the mainstream deammonification process. As the seeding for the mainstream process, performance and microbial ecology of the sidestream DEMON™ reactors have a considerable impact on the mainstream process. After an initial period of poor performance, post-seeding, both sidestream processes recovered in terms of both

performance and the associated microbial ecology (Figure 29 and Figure 30). In terms of the different microbial relative concentrations, the fraction of AMX decreased around June 2015 in both sidestream reactors, from $49 \pm 0.96\%$ and $73 \pm 3.2\%$ in June 2015 to $0.51 \pm 0.24\%$ and $0.86 \pm 0.0010\%$ in early August 2015, respectively. Recovery of AMX was observed late August 2015, achieving an average anammox relative abundance of $92.53 \pm 11.54\%$ and $76 \pm 6.9\%$ respectively around January 2015. A similar trend was observed for AOB fractions, represented by relative abundance of amoA, which decreased from $52 \pm 4.5\%$ and $56 \pm 1.7\%$ in May 2014 to $3.2 \pm 0.53\%$ and $0.80 \pm 0.18\%$ in July 2014. Recovery of AOB fractions was observed thereafter. As expected, during stable operation of the sidestream processes, the overall NOB fractions (including *Nitrospira* spp. and *Nitrobacter* spp.) were lower than that of AMX and AOB fractions. In contrast, periods of high NOB fractions coincided with periods of process upsets around early July 2015 and May 2016, such as biomass loss or excessive nitrite accumulation.

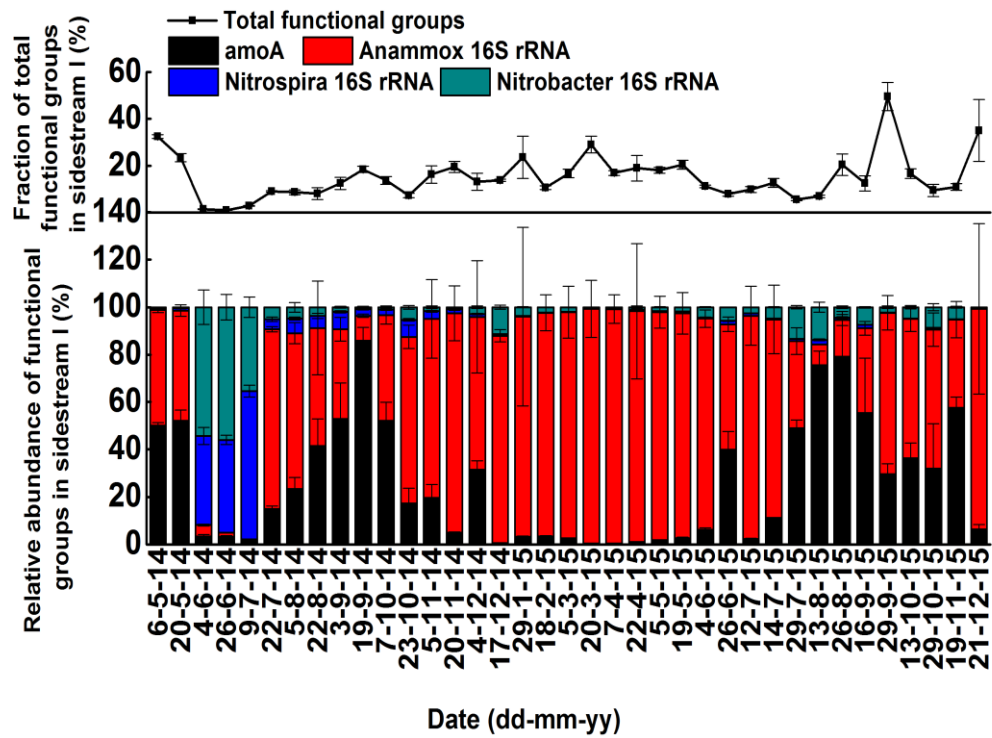


Figure 29 Relative abundance of AMX, AOB and NOB in two sidestream I DEMONTM reactor, results represent average and standard deviation of triplicate measurements.

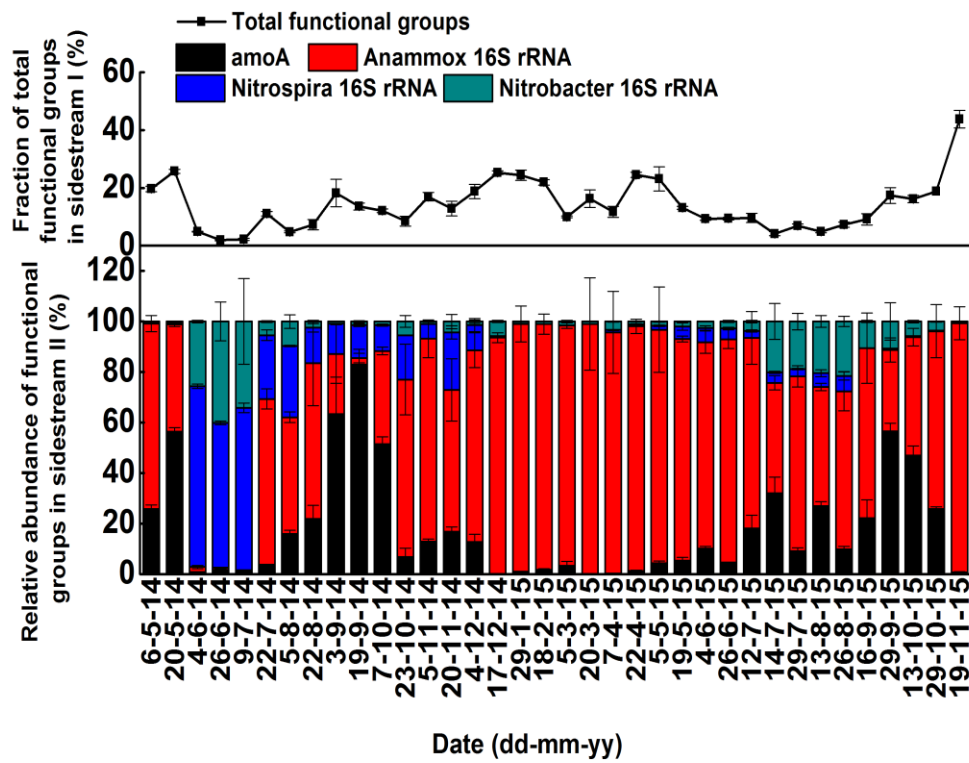


Figure 30 Relative abundance of AMX, AOB and NOB in two sidestream II DEMONTM reactor, results represent average and standard deviation of triplicate measurements

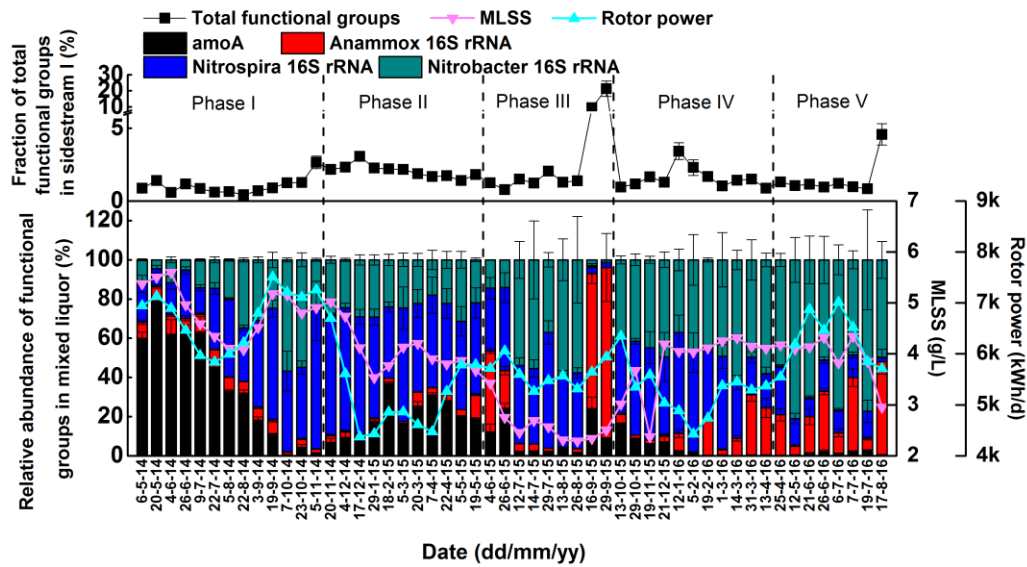


Figure 31 Relative abundance of AMX, AOB and NOB in mixed liquor of mainstream in 5 phases: (1) Only hydrocyclones; (2) Hydrocyclones and seeding from sidestream in winter operation; (3) Hydrocyclones and seeding from sidestream in summer operation; (4) Hydrocyclones and seeding from sidestream in winter operation; (5) Hydrocyclones with sidestream seeding. Results represent average and standard deviation of triplicate measurements.

6.3.2 Mainstream deammonification process

Mainstream deammonification was instituted through the installation and operation of hydrocyclones during late April, 2014. Based on qPCR analysis, the initiation of the mainstream deammonification process conditions resulted in a significant increase in the relative fractions, principally of *Nitrospira* spp. and to an extent of *Nitrobacter* spp., at the expense of AOB (Figure 31). Towards the end of phase II, the relative fractions of AOB, *Nitrospira* spp., *Nitrobacter* spp. and AMX were ($19 \pm 0.18\%$), ($47 \pm 7.7\%$), ($22 \pm 0.91\%$) and ($12 \pm 0.49\%$), respectively (Figure 3). During Phase III, after sustained seeding from the sidestream deammonification process, there was a further shift in the relative biomass fractions towards the re-emergence and

enrichment of AMX primarily at the expense of AOB and to a lesser extent at the expense of *Nitrospira* spp. (Figure 31). During phase IV and V, the mixed-liquor biomass was mainly comprised of AMX and *Nitrobacter* related NOB and to a lesser extent *Nitrospira* spp., while the fraction of AOB remained extremely low (Figure 31).

The biomass composition in the mainstream process is impacted by the sustained bioaugmentation from the sidestream process as well as the impact of the recycle from the hydrocyclone underflow (Figure 31). Imposed with the operation of the hydrocyclone with various levels of rotor power, MLSS of the mixed liquor in mainstream varied correspondingly. Retention of larger size granules could be enhanced when applying hydrocyclone with strong rotor power, while AOB was mostly confirmed to be present in the suspended biomass or small size granules. Thus, the wash out of AOB was consistently monitored corresponding to the decreased MLSS during this entire time series experiment.

The enrichment of AMX and *Nitrobacter* spp. in the mainstream is to be expected based on a sustained input of AMX from both the sidestream deammonification process and the hydrocyclone underflow (Figure 33). Given the far higher load of the biomass from the hydrocyclone underflow, the relative concentrations of AOB, *Nitrospira* spp., *Nitrobacter* spp. and AMX in the mainstream biomass (Figure 31) are expected to mirror those in the hydrocyclone underflow (Figure 33) more closely than the fractions in the sidestream process (Figure 29 and Figure 30).

6.3.3 Mechanisms of N-cycling in the mainstream process

During Phase I, the biomass fractions were in line with theoretically based biomass yields (Grady; M&E) (Figure 31). However, in response to the imposed process conditions, the relative contribution of AOB declined nearly entirely and the biomass was comprised of mainly AMX, *Nitrospira* spp. and *Nitrobacter* spp. towards the end of phase IV. This suggests a deviation from the traditional model and understanding for N-cycling in the process. All three groups, AMX, *Nitrospira* spp. and *Nitrobacter* spp. need nitrite as a substrate. There are several pathways for nitrite production, including AOB mediated NH_3 oxidation, CMX mediated NH_3 oxidation (and extracellular leakage), partial reduction of nitrate by denitrifying bacteria and recycling from the sidestream reactor.

Overall, three hypotheses are proposed to explain the monitored high NOB/AOB ratio in mixed liquor, overflow and underflow after August 2014. Nitrite looping is a phenomenon that nitrite was trapped between nitrite oxidation and nitrate reduction, where denitrifiers reduce nitrate to nitrite supplying additional nitrite for NOB. NOB could overgrow with access to nitrite from both partial nitrification and partial denitrification. Moreover, a persistently high *Nitrospira* to AOB ratio indicate that non-canonical pathways for nitrification may dominate the ammonium oxidation in the mainstream, which results from the capability of oxidation over ammonium by *Nitrospira* [87, 88]. At last, the recently reported high yield of *Nitrospira* and heterotrophic growth could also contribute to the high NOB/AOB ratio in the mainstream [17, 25].

In general, the observed performance profiles and biomass concentration trends were correlated. Significant loss of AOB was demonstrated along with the accumulation of NOB especially for *Nitrospira* spp.. Relative abundance of AOB decreased from $79 \pm 2.1\%$ in May 2014 to $0.54 \pm 0.22\%$ in October 2014, while relative abundance of *Nitrospira* spp. was enhanced from $9.0 \pm 1.8\%$ in May 2014 to $70 \pm 15\%$ in November 2014 (Figure 31). The opposite trend on AOB and *Nitrospira* spp. could be explained by the operation of the hydrocyclone. It was reported that among three different sizes of granular sludge ($< 200 \mu\text{m}$, $200 \mu\text{m} - 400 \mu\text{m}$ and $> 400 \mu\text{m}$), *Nitrospira* spp. had a higher fraction in moderate-sized ($200 \mu\text{m} - 400 \mu\text{m}$) granular sludge than that in small-sized ($< 200 \mu\text{m}$) granular [100]. Other researchers also demonstrated that concentration of *Nitrospira* spp. was enhanced with increasing granular size until $250 \mu\text{m}$, after which a decrease of fractions was observed [101]. On the other hand, presence of AOB was reported mainly to stay in the outer layer of granules and had a relatively higher fraction in smaller granules [99, 100]. However, AOB recovered gradually after December 2014 and remained at a relatively high level (average relative abundance of $23 \pm 7.5\%$ from January 2015 to June 2015). However, after June 2015 the fading of AOB was monitored once again (Figure 31). In phase III, the mainstream was operated under lower SRT, during which a rapid but short peak of AOB was observed at September 2015, followed by an abrupt reduction after October 2015. Lower relative abundance of AOB in phase III was probably contributed by low SRT condition in mainstream as well as in phase IV, while *Nitrospira* was still selectively retained during phase III and IV. Active seeding from sidestream was

introduced into mainstream in phase V, during which reduced relative abundance of *Nitrosira* spp. were demonstrated along with enhanced relative abundance of *Nitrobacter*. It was reported that *Nitrospira* are sensitive to nitrite and oxygen, substrates of NOB, thus *Nitrobacter* spp. was more favored over *Nitrospira* spp. under high dissolved oxygen (DO) and short SRT conditions [24, 107, 187]. AMX was detected since the beginning of the molecular monitoring in the mainstream, enhancement on AMX relative abundance in mixed liquor was observed in phase II. Operation of hydrocyclone could selectively retained and recycled bigger size granules, which was reported to contain higher fraction of AMX [101, 188]. Within phase IV, continuous active seeding provide sufficient activated sludge containing high fraction of AMX, which could significantly increase the relative abundance of AMX in the mainstream mixed liquor.

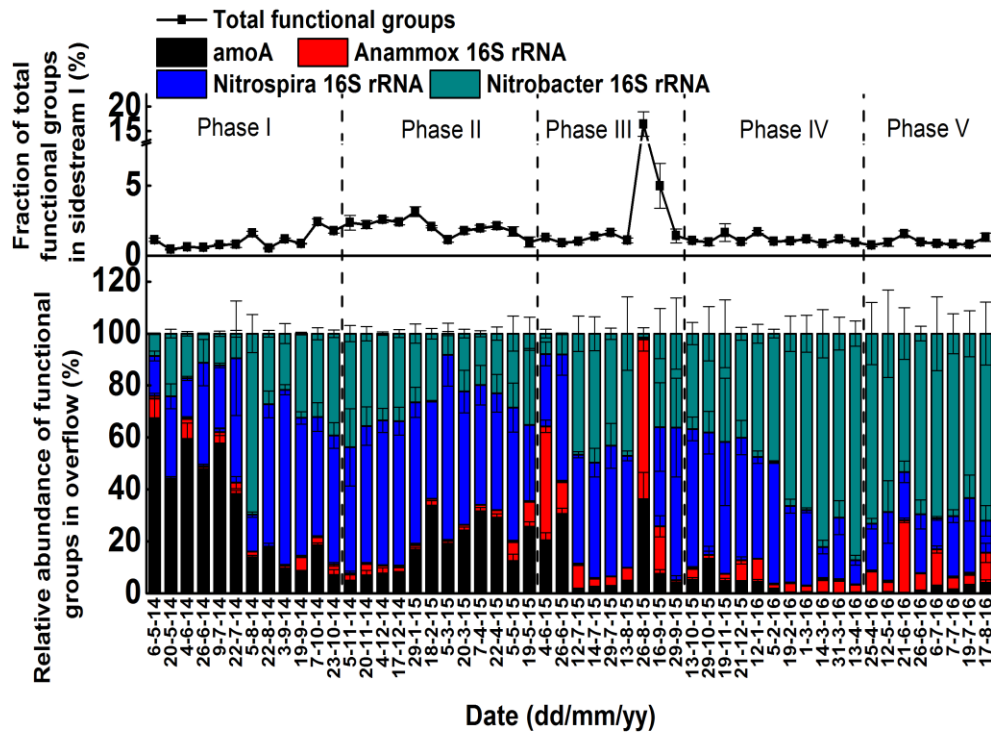


Figure 32 Relative abundance of AMX, AOB and NOB in overflow of mainstream in 5 phases: (1) Only hydrocyclones; (2) Hydrocyclones and seeding from sidestream in winter operation; (3) Hydrocyclones and seeding from sidestream in summer operation; (4) Hydrocyclones and seeding from sidestream in winter operation; (5) Hydrocyclones with sidestream seeding. Results represent average and standard deviation of triplicate measurements.

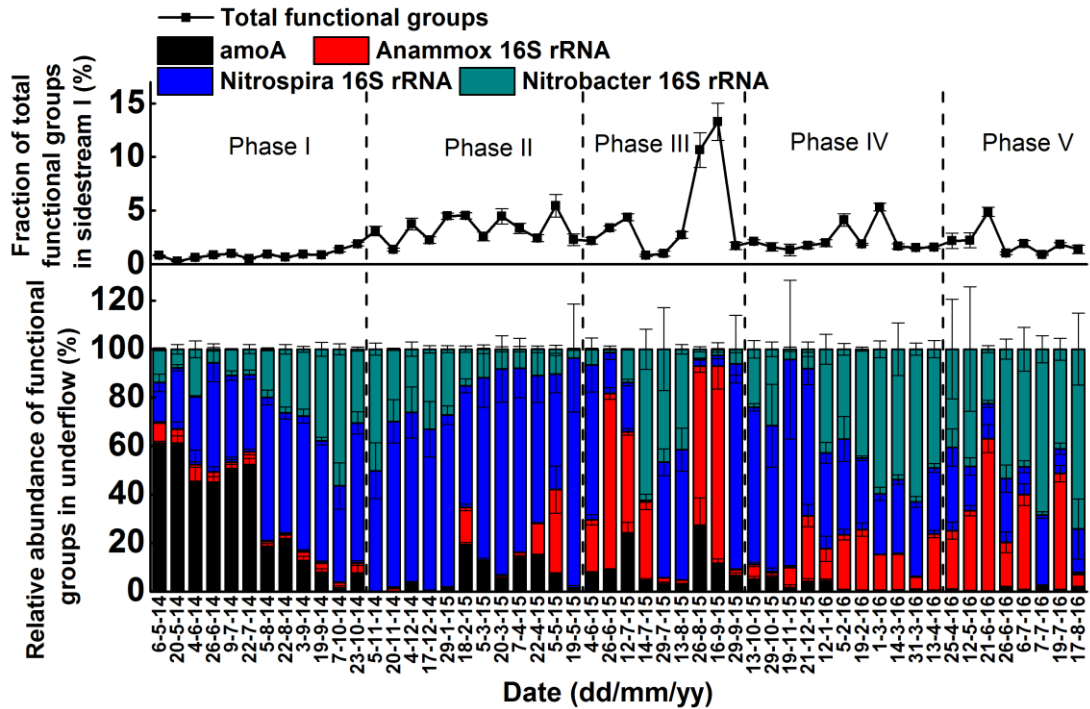


Figure 33 Relative abundance of AMX, AOB and NOB in underflow of mainstream in 5 phases: (1) Only hydrocyclones; (2) Hydrocyclones and seeding from sidestream in winter operation; (3) Hydrocyclones and seeding from sidestream in summer operation; (4) Hydrocyclones and seeding from sidestream in winter operation; (5) Hydrocyclones with sidestream seeding. Results represent average and standard deviation of triplicate measurements.

Microbial ecology in the overflow (Figure 32) and underflow (Figure 33) of mainstream were also examined in this research during this whole monitoring period.

Relative abundance of AOB was consistently lower in the underflow than in overflow.

On the other hand, *Nitrospira* and AMX had higher relative abundance in underflow than that in overflow, as discussed above that small size granules contain less fraction of *Nitrospira* and AMX [101, 188].

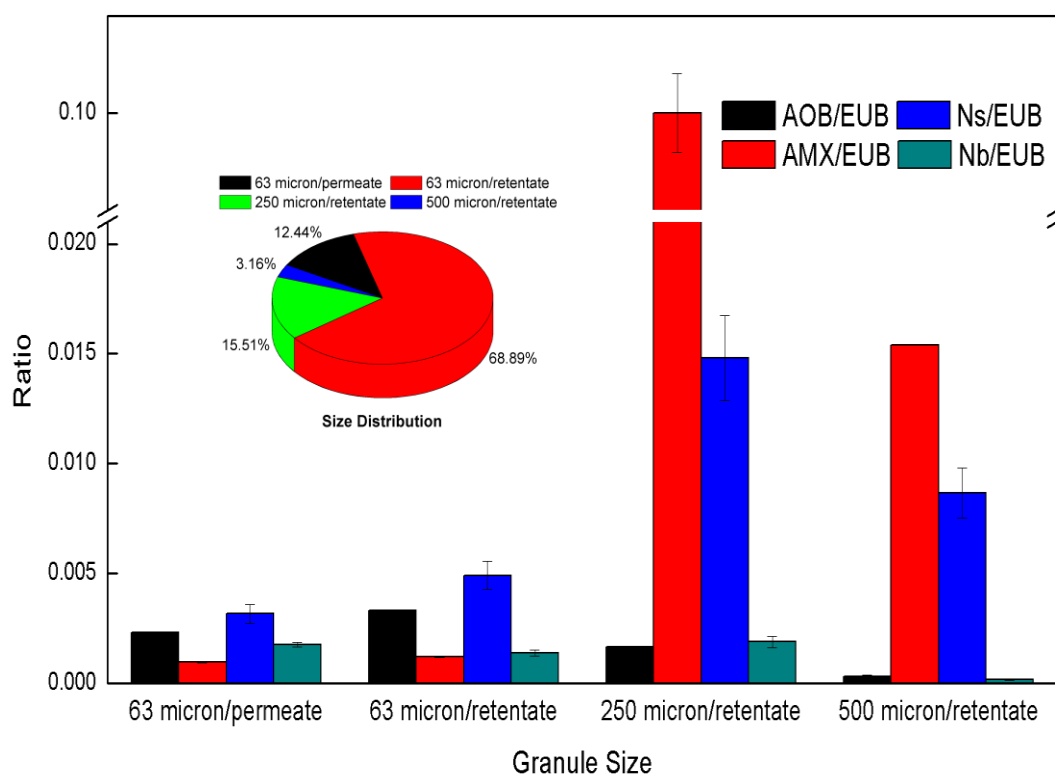


Figure 34 Microbial ecology in different size of granules at 24-Jun-15 are shown in column chart, proportions of different size granules in mixed liquor are shown in pie chart; results represent average and standard deviation of triplicate measurements.

As shown in Figure 34, different sizes of granules from mixed liquor by sieving were tested. Most of AOB (black column) were retained in the small size granules or as free cells in 63 micron/permeate. Moreover, a dramatic difference was demonstrated for AMX fractions between different size granules, relatively higher in large size granules (250 micron/retentate and 500 micron/retentate) but less abundant in small size granules (63 micron/permeate and 63 micron/retentate). *Nitrospira* spp. (red

column) was relatively abundant in all size granules, especially in large size granules. The pie chart in Figure 31 shows the ratio of different size granules in the mixed liquor sample, calculated based on total bacteria concentration. Most of bacteria are from 63 micron/retentate, in which *Nitrospira* spp. is most abundant. With operation of the hydrocyclone, granules with larger size were selectively maintained and recycled, which resulted in the out-selection of AOB and enrichment of AMX and *Nitrospira*.

6.4 Conclusions

In sum, AMX was the most abundant functional group in two parallel DEMON™ systems, demonstrating the successful startup of anammox in the sidestream. Moreover, AMX was selectively retained in the mainstream with high bioaugmentation rates from the sidestream during phases IV and V, but out selection of AOB was indicated by the fading fractions in the mainstream mixed liquor. Three hypotheses are proposed to describe the monitored high NOB/AOB ratio: (1) nitrite looping, (2) oxidation over ammonium by *Nitrospira*, (3) high yield of *Nitrospira* and heterotrophic growth. A detailed analysis of the correlation between system performance and microbial ecology is needed in future research

Chapter 7. Spectrophotometric Determination of Hydrazine in Anammox Reactor

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7.1 Introduction

ANAMMOX is a globally important microbial process of the nitrogen cycle. Hydrazine was proven the first time to be a critical intermediate in ANAMMOX at 1997 [189]. And it was further confirmed by later research that hydrazine makes dinitrogen gas and provides electrons for nitrite reduction [190, 191].

Because of the significance of hydrazine in the anammox process, a sensitive and reliable method is required for hydrazine determination in this particular case. Several methods based on different analytic techniques have been developed for the determination of hydrazine in past decades, which include spectrophotometric [90, 192], coulometric [193], titrimetric [194], voltammetric method [195] and gas chromatographic methods [196]. Because of the requirement of quick determination and the relatively low concentration of hydrazine, a spectrophotometric method is more useful for the determination of hydrazine in the anammox process.

It has been found that the determination of hydrazine by spectrophotometric method[90] is interfered by nitrite, which is the electron acceptor for anammox bacteria in all anammox reactors. It has been reported [197] that interference of nitrite (4 mg/L) can be eliminated by treating hydrazine sample(0.24 mg/L) with sulfamic acid (5 g/L). However, normally, the concentration of nitrite in anammox reactors varies between 5 mg-N/L and 50 mg-N/L, based on the operational condition, or even higher. Therefore, a more reliable method is required for the quantitation of hydrazine in the anammox process.

In this paper, based on a spectrophotometric method [90] developed by George W. Watt, an improved method is proposed for the determination of hydrazine in the presence of nitrite. The method developed in this paper has been successfully applied to the determination of hydrazine in an anammox reactor.

7.2 Materials and methods

7.2.1 Apparatus

All absorbance measurements were made using a Thermo Spectronic Genesys 20 (4001/4) spectrophotometer with 1 cm matched glass cells, except for the full wave length scanning, which was made using a Varian Cary 50 Bio spectrophotometer with 1 cm matched glass cells.

7.2.2 Reagents

All chemicals used were analytical grade reagent. A standard solution of hydrazine (100 mg/L) was purchased from Fisher Scientific. A stock solution of sulfamic acid was prepared by dissolving 2.0 g of sulfamic acid in water and diluting to 100 mL. A stock solution of nitrite was prepared by dissolving 4.9282 g of sodium nitrite in water and diluting to 100 mL. A solution of *p*-dimethylaminobenzaldehyde was prepared by dissolving 0.4 g of *p*-dimethylaminobenzaldehyde with 20 mL of ethanol and 2 mL of concentrated hydrochloric acid.

7.2.3 Determination of hydrazine

To a series of 10 mL test tubes, 9 mL of sample with hydrazine concentration between the range 0.020 mg-N/L to 1.0 mg-N/L and 1 mL of color reagent were added. Blanks

consisted of 1 mL of the color reagent and 9 mL of distilled water. The resulting mixtures were kept in dark for 20 minutes. The absorbance of the solutions were measured at 458 nm to construct a calibration graph.

7.2.4 Determination of hydrazine with nitrite and sulfamic acid

To a series of 50 mL glass beakers, a known amount of standard solution of hydrazine and stock solution of nitrite were added, after which a known amount of stock solution of sulfamic acid was added immediately and diluting to 50 mL. Before any reaction happen, the concentration of hydrazine was within the range 0.020 mg-N/L to 1.0 mg-N/L, the concentration of nitrite was within the range 5.0 mg-N/L to 50 mg-N/L, the concentration of sulfamic acid was within the range 200 mg/L to 2000 mg/L. The resulting mixture was kept in dark for 1 hour.

A sample volume of 9 mL from the above solution was analyzed by following the procedure described under the section on calibration graph.

7.3 Results and discussion

The spectrometric method developed by Watt and Chrisp [90] was based on a yellow color developed upon addition of *p*-dimethylaminobenzaldehyde to solutions of hydrazine in diluted hydrochloric acid solutions. Hydrazine reacts with *p*-dimethylaminobenzaldehyde in an acidic condition to yield a compound, i.e. a *p*-quinone structure which is yellow, and has an absorption maximum at 458 nm (Figure 35).

However, this widely used spectrophotometric method is not applied properly for the determination of hydrazine in an anammox reactor. The failure of an accurate measurement in anammox system is mainly caused by the presence of nitrite, which is a common anion in anammox and can react with hydrazine to produce dinitrogen gas. The interference of nitrite to hydrazine determination was established in Table 7. In sample 1, with 1.0 mg-N/L NO_2^- , recovery of hydrazine decreased from 100.30% to 0.49%. In sample 2, with 1.0 mg-N/L NO_2^- , recovery of hydrazine decreased from 99.89% to 5.56% and kept decreasing as the concentration of nitrite increased. As we know, nitrite concentration in anammox reactor is up to 50 mg-N/L normally, hence assaying for hydrazine without considering the interference of nitrite can no longer be trusted.

Table 7 Effect of nitrite in the hydrazine determination

Concentration of NH_2NH_2 (mg-N/L) ^a	Concentration of NO_2^- (mg-N/L) ^a	Concentration of NH_2NH_2 by <i>p</i> -DAB method (mg-N/L)	Recovery of hydrazine (%)	Standard deviation
0.1	—	0.1003	100.30	0.0018
	1.0	0.0049	0.49	0.0003
1.0	—	0.9989	99.89	0.0021
	1.0	0.0556	5.56	0.0011
	5.0	0.0361	3.61	0.0009

^a Known amount standard solution of hydrazine and stock solution of nitrite were added in beaker and diluting to 50 mL. The resulting mixture was kept in the dark for an hour before determination.

Therefore, to properly assay for hydrazine, the rapid elimination of nitrite is necessary, since nitrite can react with hydrazine. It was experimentally established that sulfamic acid can react with nitrous acid, and as products of the reaction, sulfuric acid, dinitrogen gas and water are produced.

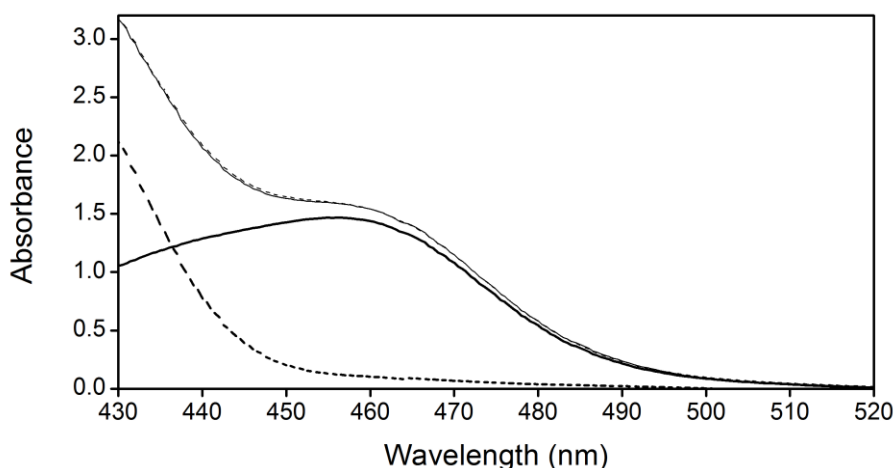


Figure 35 Absorption spectra of N_2H_4 1 mg-N/L (—), $\text{NH}_2\text{SO}_3\text{H}$ 2000 mg/L (---), N_2H_4 1mg-N/L with $\text{NH}_2\text{SO}_3\text{H}$ 2000 mg/L (—), sum of N_2H_4 1 mg-N/L and $\text{NH}_2\text{SO}_3\text{H}$ 2000 mg/L (---)

Different concentrations of hydrazine (from 0.02 mg-N/L to 1.0 mg-N/L) and nitrite (from 5.0 mg-N/L to 50 mg-N/L) were studied after treating with sulfamic acid (from 200 mg-N/L to 2000 mg-N/L). When the concentration of sulfamic acid is 200 mg-N/L, it was found that the recovery of hydrazine determination was greater than 98% with nitrite concentration under 10 mg-N/L. However, with nitrite concentration higher than 10 mg-N/L, the recovery of hydrazine determination decreased quickly to

below 60% with the increasing of nitrite concentration up to 50 mg-N/L. Hydrazine was easily reacted with presence of nitrite, when concentration of nitrite was relatively high and concentration of sulfamic acid was low. Therefore, measurements of hydrazine samples with sulfamic acid concentration up to 2000 mg/L were studied. Hydrazine was barely reacted, since nitrite was eliminated quickly and completely by high concentrations sulfamic acid. However, relatively small amount of absorbance at 458 nm was observed, when a sample with the concentration of sulfamic acid up to 2000 mg/L was determined by the *p*-DAB method (Figure 35). It was observed that absorption spectra of N_2H_4 1 mg-N/L with $\text{NH}_2\text{SO}_3\text{H}$ 2000 mg/L overlapped with the numeric sum of absorption spectra of N_2H_4 at 1 mg-N/L and $\text{NH}_2\text{SO}_3\text{H}$ at 2000 mg/L. Determinations of different concentration of hydrazine with and without 2000 mg/L sulfamic acid were studied (Figure 36). It was shown that, with the same concentration of hydrazine, sample with addition of sulfamic acid had relatively higher absorbance. The recovery of hydrazine was 102.90%, when the concentration of hydrazine was 1 mg-N/L. However, recovery at low concentration of hydrazine was extremely high, which was up to 309.21% under hydrazine concentration at 0.02 mg-N/L. To achieve reasonable determination of hydrazine, absorbance contributed by sulfamic acid must be deducted, the corrected absorbance at 458 nm with the contribution by sulfamic acid deducted is shown in Table 8. With hydrazine concentrations between 0.02 mg-N/L and 1.00 mg-N/L, the recoveries of hydrazine were within the range 97.36% to 103.61%.

Table 8 Hydrazine determination with 2000 mg/L sulfamic acid

	Concentration of NH ₂ NH ₂ (mg-N/L)	Absorbance at 458 nm	Recovery of hydrazine (%)	Standard deviation
Measurements	0.02	0.090	309.21	0.0008
	0.1	0.197	138.35	0.0011
	0.3	0.513	111.24	0.0009
	0.5	0.799	105.40	0.0018
	1.0	1.483	102.90	0.0014
Measurements be revised ^a	0.02	0.033	103.61	0.0008
	0.1	0.140	97.36	0.0011
	0.3	0.456	98.76	0.0009
	0.5	0.742	97.83	0.0018
	1.0	1.426	98.93	0.0014

^a Absorbance of hydrazine were calculated by deducting the absorbance contributed by sulfamic acid.

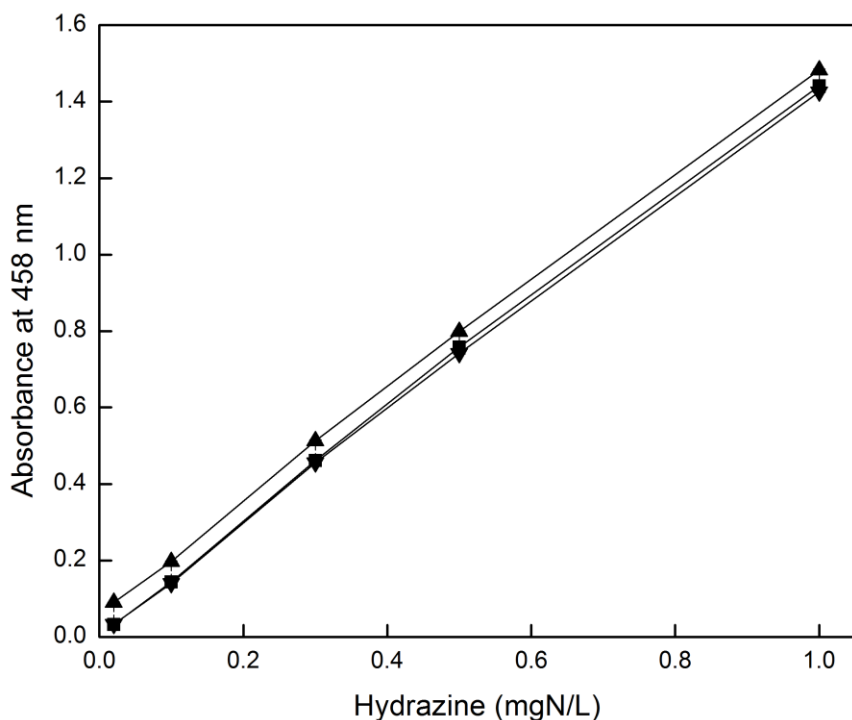


Figure 36 Absorbance of hydrazine at 458 nm “■”, absorbance of hydrazine with 2000 mg/L sulfamic acid at 458 nm “▲”, absorbance of hydrazine with 2000 mg/L sulfamic acid at 458 nm after wiping off the absorbance of sulfamic acid “▼”

7.4 Application

Hydrazine exists in anammox reactor as a critical intermediate. The application of the proposed method is evaluated with samples taken from an anammox reactor to determine the hydrazine concentration, in which concentration of nitrite is within the range 10 mg-N/L to 50 mg-N/L. Samples were filtered immediately after taken from the reactor to eliminate bacteria. Then, 8 mL filtered sample, a known amount of standard hydrazine solution and stock solution of sulfamic acid were added in 10 mL tube and diluted to 10 mL. After incubation of an hour, 9 mL of sample from 10 mL

tube and 1 mL color reagent were added into another fresh tube, which would be measured after 20 minutes' incubation. Hydrazine measured by the proposed method and its recovery was shown (Table 9). Added hydrazine showed recovery greater than 98%.

Table 9 Determination of hydrazine in anammox reactor

Samples ^a	Concentration NH ₂ NH ₂ increased by (mg-N/L)	of Concentration hydrazine by proposed method (mg-N/L)	Recovery of hydrazine (%)	Standard deviation
Sample 1	—	0.023	—	0.0010
	0.1	0.122	99.76	0.0008
	0.3	0.322	99.84	0.0012
Sample 2	—	0.031	—	0.0014
	0.1	0.130	99.30	0.0010
	0.3	0.331	100.14	0.0020
Sample 3	—	0.027	—	0.0008
	0.1	0.126	98.85	0.0007
	0.3	0.325	99.30	0.0010

^a Samples were withdrawn from anammox reactor and filtered.

7.5 Conclusions

The proposed method is an easy, selective, rapid, sensitive and reliable spectrophotometric method. The molar absorptivity of the developed method is $4.1 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$. The detection limit of hydrazine for the proposed method is $4.37 \text{ }\mu\text{g-N/L}$. The method was applied for the determination of hydrazine in an anammox reactor. The results show that this method is suitable for determination of hydrazine in the presence of nitrite.

Chapter 8. Conclusions

8.1 Key findings

8.1.1 Enrichment of mainstream anammox reactors can be achieved under both ambient temperature and low temperature

Lab-scale mainstream MBBR was successfully enriched and achieved a stable and satisfactory process performance by coupling with the sidestream MBBR at both ambient temperature and low temperature. As revealed by the microbial ecology, in both ambient- and low-temperature mainstream reactors, suspended biomass from the sidestream MBBR led to the enrichment of AMX in the mainstream MBBR during bioaugmentation yet did selectively wash out *Nitrospira* spp. related NOB in the ambient-temperature mainstream reactor. However, in the low-temperature anammox mainstream reactor, an effective inhibition of *Nitrospira* spp. was unintentionally achieved by increasing the concentration of influent ammonium, and remarkably high fraction of AMX was accumulated in the biofilm biomass in the low-temperature anammox reactor. Moreover, the presence of *Chlorobi* and *Chloroflexi* related bacteria was detected in both mainstream reactors, which are commonly reported co-existing microbes in the anammox system. It might be partly contributed by the exposure of system to light and further research to clarify the mechanism and inhibition of *Chlorobi* are required. Furthermore, the enriched mainstream MBBR was evaluated by imposing wet weather conditions, thus the reliability under some extreme conditions could be confirmed. These results are crucial to show the applicability of mainstream deammonification even in low temperature condition, allowing for the significant cost savings through anammox.

8.1.2 Organic carbon has a reversible negative impact on the mainstream anammox MBBR

After imposing synthesized organic carbon, the performance of anammox was significantly inhibited yet fully recovered after the imposed organic carbon was terminated. However, despite the suppression of anammox, a good average total nitrogen removal over 70% could be still be achieved with the contribution of denitrification. Results of SIP and 16S rRNA gene sequencing revealed the incapability of anammox to assimilate acetate, the community assimilating ^{13}C -labeled acetate was mainly assigned to denitrifiers. Though fractions of denitrifiers were not significant and remained inactive when running a reactor with no organic carbon condition, they could be quickly boosted after sufficient supply of organic carbon. These results demonstrate that the mainstream anammox process as tested in this study was resilient to varying influent loads of organic carbon, it is critical to show the potential of anammox application in real wastewater conditions.

8.1.3 Long-term monitoring of full-scale mainstream and sidestream deammonification processes

In this full-scale WWTP, anammox process was firstly initiated in the sidestream, and bioaugmentation were implemented afterward to enrich AMX in the mainstream. Though two parallel sidestream reactors had failures in the startup, a dominant fraction of AMX was still achieved. With respect to the mainstream, by retaining large size granules containing a relatively high fraction of AMX, AMX was successfully achieved especially during the period with active seeding from the

sidestream system. However, the relative contribution of AOB declined nearly entirely in response to the implemented system conditions, which led to the shift of microbial ecology to mainly AMX, *Nitrospira* spp. and *Nitrobacter* spp.. In sum, Anammox bacteria was selectively retained in the mainstream with high bioaugmentation rates from the sidestream during phase IV and V. Significant high fraction of anammox bacteria was observed in big size granules (250~500 μm and $>500 \mu\text{m}$), indicating that selectively retaining of big size granules could be beneficial to the enrichment of anammox bacteria.

8.1.4 Hydrazine measurement in anammox system

As a unique intermediate of anammox process, hydrazine could be applied as an indicator of anammox activity. However, limited by the negative influence of nitrite, current applied spectrophotometric method of determining hydrazine could rarely be applied to precisely measure the level of hydrazine concentration in an anammox reactor. Based on regular spectrophotometric method of determining hydrazine, a more reliable method was proposed to achieve a rapid and sensitive hydrazine measurement with in the presence of nitrite. As a unique intermediate of anammox reaction, the level of hydrazine concentration could be applied as an effective and efficient indicator to reveal the activity of AMX, thus indicating the significant value of this new proposed method.

8.2 Future directions

With the critical economic and environmental potential of anammox process, more

and more attention has been attracted to the application of the anammox process for nitrogen removal from domestic WWTP. Considering that the most of the nitrogen loading of WWTP is located in the mainstream, significant efforts have been made by many researchers to move the implementation of mainstream application forward. However, several substantial obstacles still need to be solved. By (1) suppressing the activity and selectively washing out NOB, *Nitrospira* spp. especially, (2) improving the feasibility and adoption of anammox application under low and variable temperature conditions, and (3) enhancing the activity of AMX with the presence of organic matter, we can reach a better understanding of anammox applications in real WWTP and achieve the high economic and environmental potential of the anammox process even under extreme conditions.

In addition, BNR processes harbor a great variety of microorganisms, within which bacteria play a crucial role in the biological removal of pollutants. Very limited information on the microbial community structure of these processes was available until a few years ago. Indeed, the use of advanced microbiological techniques of NGS and bioinformatics to explore the microbial diversity of bioreactors can support the understanding, design and optimization of more complex engineered biochemical processes.

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Appendix

Additional projects

Z. Li, H. Ying, K. Chandran “Sidestream deammonification startup - process controls.”

Z. Li, H. Ying, K. Chandran “Mainstream deammonification process startup.”

Z. Li, H. Clippel, Q. Zhang, K. Chandran “The microbial ecology of a deammonification system for digester supernatant pretreated with thermal hydrolysis”

Z. Li, G. Johnnie, C. Bott, K. Chandran “The microbial ecology of a anammox MBBR operated as a polishing step for BNR system.”

Z. Li, S. Michael, C. Bott, K. Chandran “The microbial ecology of a conventional BNR system.”

Presentations

Z. Li. “Enrichment and Characterization of an Anammox Moving Bed Biofilm Reactor (MBBR) to Treat Mainstream Wastewaters..” WEF/IWA. Denver, July. 2016.

Z. Li. “Possibility of *Nitrospira* spp. mediated complete ammonia oxidation (comammox) in a full-scale mainstream deammonification process.” WEFTEC. New Orleans, Sept. 2016.

Z. Li. “Effect of Organic Matter on the Performance and Microbial Ecology of a Mainstream Anammox Process.” WEFTEC. Chicago, Oct 2017.